STUDIES ON THE MECHANISM OF ACTION OF WY 23675,
A NOVEL HYPOGLYCAEMIC AGENT.

by

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The mode of action of the novel hypoglycaemic agent, Wy 23675, has been compared with that of the sulphonylurea, tolbutamide, and that of the biguanide, phenformin.

Wy 23675 has been shown, like tolbutamide, to stimulate insulin release, but it has also been shown to have an insulin-independent hypoglycaemic activity. Wy 23675 lowers blood glucose levels in both normal and alloxan-diabetic rats in contrast to phenformin, which does not affect blood glucose levels in the normoglycaemic rat.

Wy 23675 has been shown to stimulate glucose utilization in vivo independent of any effect on insulin release, whereas tolbutamide is only active when it can stimulate insulin release. Phenformin stimulated glucose utilization in vivo, but only in hyperglycaemic conditions.

Phenformin stimulated glucose utilization by hemidiaphragms at a lower concentration when they were prepared from alloxan-diabetic rats than from normal rats. Phenformin caused a decreased glycogen content and an increase in lactate production in the hemidiaphragm, suggesting an increase in glycolysis and a possible inhibition of lactate oxidation. Phenformin, in high concentrations, stimulated glucose utilization by adipocytes prepared from normal and alloxan-diabetic rats.

Wy 23675 stimulated glucose uptake into isolated hemidiaphragms and adipocytes prepared from normal and alloxan-diabetic rats. In hemidiaphragms, Wy 23675 caused an increase in glycogen synthesis with no effect on lactate or pyruvate production. The measured increase in glucose uptake could not be solely accounted for by the measured increase in glycogen content, suggesting an increase in glucose oxidation. The possibility that Wy 23675 could activate pyruvate dehydrogenase is discussed.

Both phenformin and Wy 23675 inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but not from xylitol in hepatocytes prepared from fasted, normal or alloxan-diabetic rats.
these drugs to inhibit gluconeogenesis than did hepatocytes from alloxan-diabetic rats or hepatocytes from fasted, normal rats which were stimulated by glucagon.

The pattern of hepatic metabolite levels following phenformin treatment of alloxan-diabetic rats showed a negative cross-over between 3-phosphoglycerate and dihydroxyacetone phosphate. Measurement of appropriate metabolite ratios showed a more reduced state in the cytosol and mitochondria and these redox changes are discussed.

Wy 23675 treatment resulted in a negative cross-over between malate and phosphoenolpyruvate indicating a possible effect on the enzyme phosphoenolpyruvate carboxykinase, although this was not supported in a preliminary in vitro experiment.
Dedication.

I dedicate this work to my parents to whom I owe so much and who have always shown a lively interest in my work and also to my wife, Anne, who has spent many a long evening listening to me bashing my typewriter into some sort of submission.
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Abstract

Dedication

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This thesis compares the hypoglycaemic effects of a novel agent, Wy 23675, with those of the two main clinically used groups of oral hypoglycaemic agents - the sulphonylureas, as represented by tolbutamide, and the biguanides, as represented by phenformin.

Drugs can cause a lowering of blood glucose levels by a variety of mechanisms. They may act indirectly by stimulating insulin release or they may cause an increase in glucose utilization and/or inhibit gluconeogenesis. They may also act by preventing the absorption of glucose from the gut or by lowering the renal threshold for glucose so that glucose is cleared in the urine.

**Insulin**

Insulin has profound effects on carbohydrate, protein and fat metabolism in liver, muscle and adipose tissue. The net result of these effects is a reduction in blood glucose, amino acid and free fatty acid levels and an increase in glycogen, fat and protein synthesis.

**Insulin Effects In The Liver.**

In the liver, insulin affects carbohydrate metabolism by inhibiting catabolic processes and stimulating glycogen synthesis (1). Insulin can suppress gluconeogenesis, but only in circumstances where it is stimulated. For example, insulin has no effect on basal rates of gluconeogenesis from different substrates in isolated hepatocytes, but it does inhibit the glucagon stimulation of gluconeogenesis (2,3). Similarly, insulin has no effect on the basal activities of the key enzymes involved in gluconeogenesis and glycolysis (eg: glucokinase, phosphofructokinase, pyruvate kinase, fructose bisphosphatase (4)), but does counteract the effects of glucagon on these enzymes (eg: insulin counteracts the inhibition of pyruvate kinase by glucagon (5)). It has also been shown that insulin reverses all glucagon-induced changes in metabolite levels involved in gluconeogenesis, which suggests a similar site of action (3). Also, in alloxan-diabetic rats, where rates of gluconeogenesis are markedly elevated, insulin has inhibitory
carboxylase, phosphoenolpyruvate carboxykinase, fructose bisphosphatase and glucose-6-phosphatase) and increases the levels of glycolytic enzymes (e.g., glucokinase, phosphofructokinase and pyruvate kinase) (8). Thus, the effects of insulin in the liver are to decrease glucose production.

Insulin Effects In The Muscle.

In muscle, glucose transport is the principle rate-limiting step for glucose utilization; insulin accelerates this process (9-13). Insulin stimulates glycogen formation in muscle and is able to do so even when bound to dextran and unable to enter the cell (14). The effect of insulin on glucose transport has been separated from its effect on glycogen synthesis (15). Insulin stimulates glucose oxidation to carbon dioxide, glycogen synthesis and glycolysis (16); the effects on glycolysis being independent of the effect on glucose transport (17, 18). Insulin appears to stimulate the synthesis of all proteins in muscle (19). It stimulates the incorporation of amino acids into protein (20, 21) and this effect is independent of the stimulatory action on the transport of certain amino acids (22, 23) - insulin stimulates the incorporation of all natural amino acids into protein, including those formed intracellularly (24). Insulin reduces plasma amino acid levels, in part at least, through suppression of their release from muscle (25). Thus, in muscle insulin stimulates glucose uptake and utilization and protein synthesis, and reduces proteolysis and amino acid release.

Insulin Effects In Adipose Tissue.

In adipose tissue, insulin enhances glucose transport (26, 27) and increases carbon dioxide production from glucose as well as the synthesis of glyceride-glycerol, fatty acids and glycogen (27) - this is independent of its effect on glucose transport (28-30). Insulin stimulates amino acid transport into fat cells (31) and increases their incorporation into proteins (32). As well as stimulating glycerol and fatty acid synthesis,
stimulation of pyruvate dehydrogenase (33-35). Thus, the effects of insulin in adipose tissue are to enhance glucose uptake and utilization, to promote glycerol and fatty acid synthesis, while inhibiting the stimulation of lipolysis, and to increase amino acid incorporation into protein.

Mechanism Of Insulin Action.

The mechanism by which insulin exerts its biochemical effects remains unclear. Several insulin-responsive enzyme systems have been linked to the regulatory influence of cyclic adenosine-3',5'-monophosphate (cAMP) - for example, glycogen synthetase and phosphorylase in muscle (36, 37), liver (38, 39) and adipose tissue (40, 41), and the hormone-sensitive lipase in adipose tissue (42, 43). Thus, insulin may act by altering intracellular levels of cAMP. Support for this concept comes from reports showing that insulin inhibits the rise in cAMP due to submaximal concentrations of hormones opposing insulin action - for example, catecholamines and glucagon in the liver (44-46) and adipose tissue (47, 48). It has also been shown that insulin inhibits adenylate cyclase in plasma membranes of liver (49, 50) and stimulates phosphodiesterase in liver and fat cell homogenates (51-59). However, other groups have found that under certain conditions insulin had no effect (60-63) or increased (64) cAMP in adipocytes while inhibiting lipolysis. Also, in muscle cAMP levels have been observed to remain constant (65) or increase (66) in the presence of insulin, while glycogen synthetase activity was stimulated. Recent evidence suggests that the effects of insulin on hepatocyte glucose output can be dissociated from effects on intracellular cAMP. It has been reported that insulin decreases basal glucose output in liver slices incubated in a high K+ medium with no effect on cAMP (67). Also, in isolated hepatocytes insulin antagonized the increased rates of gluconeogenesis due to adrenaline (68), which has been demonstrated to act on this process by a cAMP-independent mechanism (69, 70). Insulin action on gluconeogenesis has been shown to be unaffected, while its effects on cAMP were abolished in the absence of
whereas the antilipolytic action does not.

More recently, a unified hypothesis of insulin action involving intracellular Ca++ flux has been proposed (72-75). Basically, it proposes that the primary action of insulin is to trigger the release of plasma membrane-bound Ca++ into the cytoplasm, which results in a rapid rise in intracellular free Ca++ followed by an increased uptake into mitochondria and, perhaps, endoplasmic reticulum stores. The increased cytoplasmic and mitochondrial Ca++ concentration is purported to mediate the actions of insulin on membrane transport and intracellular enzymes, which stimulate glycogenesis and lipogenesis and decrease lipolysis and glycogenolysis.

In support of this concept, it has been shown that several effects of insulin or agents that mimic its actions in muscle require Ca++ in the medium (76-80); increased Ca++ flux has been observed in various conditions mimicking insulin action (73, 81-85); and insulin has been shown to have direct effects on Ca++ flux (86-92). However, this model cannot explain all the actions of insulin. For example, it seems that the response of certain insulin-sensitive enzyme systems to Ca++ is in the opposite direction to that of insulin (93-97).

**Sulphonylureas**

The hypoglycaemic effects of the sulphonylureas were originally thought to be entirely due to the stimulation of insulin release from the pancreas (98-101). This conclusion is supported by studies which showed that there was an increase in the 'insulin-like' activity of pancreatic venous blood following intravenous injection of tolbutamide - Goetz and Egdahl (99) assayed this activity by the hypoglycaemic response in mice. Pfeiffer et al (100) found that three hours after administration of tolbutamide to calves the extractable insulin content of the pancreas was reduced by about 75%; Root (101) observed similar changes in the dog.
humans (102-105), cats (106), or dogs (107-109). Similarly, sulphonylureas do not reduce blood glucose concentrations in alloxan-diabetic rats (110-113), rabbits (114), or dogs (115).

Direct effects of sulphonylureas on the pancreatic β-cells have been shown. The cells lose their granules under the influence of these agents (116) and the amount of insulin recoverable from the pancreas falls in parallel with the degranulation (117). Furthermore, electron microscope studies have shown that the β-cell granules are contained in membranous sacs which move under the influence of the sulphonylureas to the cell membrane, where they expel their contents into the pericellular fluids by a process called emiocytosis (118).

However, although acute treatment with sulphonylureas does clearly cause an elevation of plasma insulin levels (119-121), it has been shown that chronic oral therapy with sulphonylureas can reduce blood glucose levels without increasing absolute circulating insulin levels (122-125). It has also been shown that plasma glucose concentration can be lowered by sulphonylureas without a rise in plasma insulin concentration when given acutely to dogs (126). Also the hypoglycaemic response to an increase in plasma insulin due to the administration of sulphonylureas is preceded by a lag period, and it continues after insulin values have returned to basal levels (119). These results cannot be explained by an inhibition of glucagon release as sulphonylureas have no effect on this (127), although there is some evidence to suggest that they can inhibit catecholamine release (128). Loubatieres et al (129) have put forward the proposal that the effects are due to a 'secretagogue amplifying' effect of the sulphonylureas.

Sulphonylurea Effects On Glucose Absorption From The Gut And On The Renal Threshold For Glucose.

Sulphonylureas have been reported by some workers to have no effects on (130-132) and by others to inhibit (133) glucose absorption from the
responsive diabetics is accompanied by decreased glucosuria (134,135).

Other Effects Of Sulphonylureas.

Very few other direct positive effects of sulphonylureas other than those on the pancreas have been reported, which could contribute to their hypoglycaemic effects. Tolbutamide, in varying concentrations, has been reported to increase (136), inhibit (137), and to have no effect on (138-140) glucose uptake by diaphragms from normal rats. Some workers have found that glycogen synthesis was increased (141) and others that it was decreased (142,143) by sulphonylureas.

Glucose uptake by adipose tissue (144) and brain (145) has not been found to be affected by sulphonylureas, but it has been claimed that the incorporation of $^{14}$C from isotopic glucose into fatty acids was inhibited (146). Several workers have reported antilipolytic effects on adipocytes (147-152) and this action could lower blood glucose levels by decreasing the available plasma free fatty acids for oxidation by muscle, thus increasing muscle glucose oxidation and lowering of blood glucose levels (153).

In the liver, sulphonylureas cause a rise in glycogen content (154), although this is dependant upon the presence of some intrinsic or extrinsic insulin (155). Sulphonylureas have been shown to diminish glucose release from the liver both in vitro (156) and in vivo (157). Tolbutamide has also been shown to inhibit the conversion of fructose to glucose in the livers of diabetic subjects (104). Sulphonylureas inhibit intrahepatic triglyceride lipase (158,159): this hormone-sensitive enzyme is thought to contribute to the regulation of gluconeogenesis, perhaps by regulating the flow of energy and regulatory acetyl CoA units from stored fatty acids, so that inhibition of the lipase could contribute to blood sugar lowering through inhibition of gluconeogenesis. Sulphonylureas have also been shown to limit the movement of substrate anions across the inner membrane of hepatic mitochondria (160,161) and other workers (162) have concluded...
greatly dependant upon anion movement into and out of mitochondria, such an action of sulphonylureas in the liver could help to reduce blood glucose levels.

Sulphonylureas have been shown to affect transmembrane Ca\(^{++}\) fluxes in several tissues (133, 135); these effects, as with insulin, may play a part in the hypoglycaemic activity of these agents.

In summary, sulphonylureas elevate plasma insulin levels when given acutely, but are capable, with chronic administration, of reducing blood glucose concentrations without affecting plasma insulin levels. Most of the in vitro effects of sulphonylureas require the presence of insulin, so that their in vivo capability of reducing blood glucose concentrations without affecting plasma insulin levels is probably due to an ability to potentiate some of the metabolic effects of insulin.

**Biguanides.**

Guanidine was shown to be hypoglycaemic following a rise in its concentration in the blood stream in 1918 (163). Various chemical modifications of the molecule were attempted and it was shown that a free guanidine group was necessary for the hypoglycaemic effect (164). The general formula for all the hypoglycaemic derivatives of guanidine is:

\[
\begin{array}{c}
\text{H} \\
\text{R} \\
\text{N-C} \\
\text{NH}_2 \\
\text{NH}
\end{array}
\]

The biguanides are the only guanidine derivatives which have been significantly used in the treatment of diabetes. Phenformin, which was first synthesized in 1929 has been studied in man (165) and was introduced in the U.S.A. for the treatment of diabetics some thirty years later. The other biguanides most commonly used in the treatment of diabetes are buformin and metformin. However, with the recent awareness of the lacticacidosis associated with biguanides, phenformin has now been withdrawn from the United States market and their use elsewhere appears
Very large doses of biguanides are necessary to lower blood glucose levels in normal rats and man (166, 167), whereas relatively small doses are required to lower blood glucose levels in diabetic man (168-172) and in experimentally-induced diabetic animals (167, 173, 174).

Biguanides tend to become concentrated in certain organs in the body. Rats given radiolabelled phenformin have nearly all the radioactivity in the digestive tract and liver and almost the whole dose is excreted by the kidneys within 24 hours (175, 176). However, these results do not necessarily indicate a selective concentration of the active drug, as almost all the radioactivity in the rat liver can be attributed to metabolic products of the biguanide (176).

There is considerable species variation in the extent of the metabolism of phenformin and this is largely reflected in its degree of hypoglycaemic activity. Phenformin is almost completely metabolized in the rat liver, mainly to p-hydroxyphenformin glucuronide (177-179), while in the guinea-pig, an animal which is very sensitive to the hypoglycaemic effects of phenformin, the extent of its metabolism in the liver is very much less (180, 181). p-Hydroxyphenformin is not an active hypoglycaemic agent (177, 182) and Cook et al (180) have shown that guinea-pigs treated with 3,4-benzpyrene, an inducer of microsomal hydroxylation, had a decreased hypoglycaemic response to phenformin. They also showed that rats treated with Lilly 18947, an inhibitor of hepatic microsomal hydroxylation, had an enhanced hypoglycaemic response to phenformin (180). Similarly, phenformin
metabolism of phenformin in the rat liver plays an important role in its relative lack of hypoglycaemic effect in the rat.

Biguanides And Insulin.

Unlike the sulphonylureas, the biguanides do not stimulate insulin release from the pancreas (183-185). However, there is some evidence that the presence of insulin is necessary for some of the effects of biguanides such as the increased peripheral uptake of glucose (167, 186-188). In contrast to this suggestion other workers have shown that biguanides can increase glucose uptake into isolated muscle without the addition of insulin (189, 190). Biguanides have also been shown to possess hypoglycaemic activity in both alloxan-diabetic (167, 173, 174) and pancreatectomized animals (191, 192). Thus, insulin is probably relatively unimportant to the hypoglycaemic effects of the biguanides.

Effects Of Biguanides On Intestinal Glucose Absorption.

Biguanides have been shown to markedly inhibit the absorption from the intestine of glucose, amino acids, lipids, ions and water (193-204). As biguanides have also been shown to accumulate in the intestine (175, 176 it has been suggested that part of their hypoglycaemic action may be through inhibition of intestinal glucose absorption. Gardner and Langslow (204) have shown that phenformin is tightly bound within the mucosal cells of the intestine and have suggested this as the site of action of phenformin on intestinal glucose absorption. Other workers (205, 206) have shown a reduction in intestinal ATP and this could account for the inhibitory effects of biguanides on intestinal glucose absorption. However, since several other biguanides inhibit oxidative phosphorylation yet have no hypoglycaemic properties (207) this may be irrelevant to the hypoglycaemic effect of phenformin. While diminished glucose absorption would restrict the increase in blood glucose concentration following a meal, it would not account for a hypoglycaemic effect in the
Effects Of Biguanides On Mitochondrial Respiration And On Gluconeogenesis.

Biguanides are potent inhibitors of mitochondrial respiration and oxidative phosphorylation (189,190,208-215). Thus, the concept was introduced that biguanides could inhibit gluconeogenesis by reducing cellular supplies of adenosine triphosphate - ATP (189,190,209). However, this hypothesis is very tenuous for several reasons:

1. The correlation between potency as respiratory inhibitors in vitro and hypoglycaemic activity in vivo is very poor (207).
2. The concentrations of biguanide required for respiratory inhibition are several orders of magnitude greater than those observed in tissues with the usual therapeutic doses in vivo (178,207,216-219).
3. The theoretical ratio of high energy phosphate required to glucose produced is 6:1 , 2:1 , and 1.2:1 for gluconeogenesis from lactate, dihydroxyacetone and xylitol, respectively. Thus, the observation that phenformin decreases the rate of gluconeogenesis from both lactate and dihydroxyacetone to about the same extent and has no effect on gluconeogenesis from xylitol (181) suggests that the effect of phenformin on gluconeogenesis is not directly related to energy requirements.
4. Other workers have found that the inhibition of gluconeogenesis by biguanides does not correlate with a measured drop in ATP levels (220).
5. Inhibition of gluconeogenesis in liver (221-223) and stimulation of glucose uptake into skeletal muscle (224) has now been observed in vitro at concentrations of biguanide too low to be associated with inhibition of mitochondrial respiration.
6. In the intact animal or in the patient, biguanides in therapeutic doses do not restrict hepatic oxygen uptake (225) or overall carbon dioxide production from glucose (226,227). On the contrary, Unger et al (228) have reported a stimulation of tissue respiration, while metformin has not been found to inhibit tissue respiration even at high doses (229).
normal rats (230) and man (226, 227) and there is direct evidence of increased glucose uptake by muscle, as shown by human forearm studies (183, 186). Losert et al (230) have reported an increased oxidation of glucose to carbon dioxide and an increase in blood lactate concentrations. Searle and Cavalieri (227) report an increase in glucose turnover in normal human subjects due to increased gluconeogenesis from lactate. They suggested that the increased gluconeogenesis was due to the increased lactate availability, following the increase in glucose utilization. They found no decrease in blood glucose levels in the normal man (227, 231), while biguanides did lower blood glucose levels in diabetic man (168-172). They explained this by suggesting that in normal man the increased peripheral glucose utilization was compensated for by increased gluconeogenesis from lactate, while in diabetic man gluconeogenesis was already maximally stimulated and could not be further increased by the increased availability of lactate, thus leading to a fall in blood glucose levels in diabetic, but not normal man. However, this hypothesis can be criticized on several grounds:–

1. The pigeon has been shown to be sensitive to the hypoglycaemic effects of phenformin, but in vitro pigeon muscle appears to be unaffected by phenformin (232); so it is unlikely that theories for the peripheral action of phenformin could explain its hypoglycaemic effects in the pigeon.

2. Phenformin has been shown to inhibit gluconeogenesis in both high concentrations (180, 181, 190, 209, 221, 233-237) and at concentrations associated with therapeutic administration of the drug (221-223); the latter concentrations being too low to be associated with inhibition of mitochondrial respiration. Furthermore, it has been shown that phenformin can inhibit gluconeogenesis by livers from diabetic rats at lower concentrations than those required for normal rats and Connon (238) has suggested that this may offer an explanation for the
being inactive in normal rats and man.

3. Recent work by Dietze et al (239) has shown an inhibition of hepatic
gluconeogenesis in normal volunteers following five days pretreatment
with phenformin (3 x 50mgs daily). However, they also showed an increase
in hepatic venous concentration ratio of $\beta$-hydroxybutyrate:acetoacetate,
indicating the possibility of interference with oxidative phosphorylation.
Lyngsoe (240) has also shown a decreased gluconeogenesis in normal
starved subjects treated with phenformin. These results support the
in vitro findings of inhibition of hepatic gluconeogenesis.

Effects Of Biguanides On Adipose Tissue.

Biguanides tend to favour weight loss and have been shown to have a
beneficial effect in human obesity (241-243), so there has been considerable
interest in their effects on fat metabolism. These effects on weight loss
could not be attributed to anorexia (242,243).

Phenformin therapy in obese diabetics has been shown to result in reduced plasma levels of free fatty acids, cholesterol and triglycerides
(244-249). In vitro, phenformin and other biguanides have been shown to
inhibit lipolysis (147-149,250,251). However, for the most part, the
antilipolytic effect of phenformin has only been demonstrated with
concentrations of the drug, which are higher than those normally observed
following treatment. Thus, the clinical significance of this action is
probably limited.

Most workers report inhibitory effects of biguanides on lipogenesis
(252-256) and these effects have been observed in concentrations similar
to those seen in the plasma with therapeutic administration of the drug
(255). However, some workers report an increase in glucose uptake and
utilization (256-260), while others have shown that phenformin depressed
glucose uptake and utilization (251,261,261).

These conflicting results and the often high doses of biguanide
required to achieve them, makes the analysis of biguanide action in adipose
tissue and its importance in terms of their hypoglycaemic effects difficul
lipolysis could lead to an improved glucose tolerance. However, as has been discussed, the antilipolytic action of phenformin only occurs in vitro with high concentrations of the drug.

Mechanism Of Action Of Biguanides.

The mechanism of action of biguanides at the cellular level has been investigated in some detail by several groups of workers. As has already been stated, it was proposed that biguanides act by inhibiting mitochondrial respiration and oxidative phosphorylation (189, 190, 208-215). However, for the reasons given earlier it is very unlikely that this concept can explain the hypoglycaemic actions of biguanides.

More recently, both Schafer (263) and Davidoff (264) have proposed theories to explain the actions of biguanides based on their binding to membranes.

Schafer's theories are based on the fact that most processes, which are sensitive to the biguanides, are either directly localized in membranes or involve membrane-linked reactions as rate-limiting steps. Biguanides have a positive charge and Schafer has shown that, in binding to membranes, they cause a positive shift in the electrostatic surface potential (265, 266-268), leading to an inhibition of proton translocation (235, 266, 268). This may be of significance in gluconeogenesis on the flux of substrate anions across mitochondrial membranes, as anion uptake is driven by the electrochemical gradient of protons across the membrane via coupling to the energy-linked uptake of inorganic phosphate (269-271).

Davidoff has shown that low concentrations of biguanides enhance Ca++ uptake in a concentration-dependent manner (272) and he has suggested that enhancement of Ca++ uptake is the basis of biguanide inhibition of gluconeogenesis (264). Biguanides, in low therapeutic doses, only lower blood glucose levels in situations where the net glucagon/insulin signal to the liver is markedly in favour of glucagon [i.e. diabetes (169) or prolonged starvation (273)]. The proposed
subcellular basis for the inhibition of the glucagon signal to the liver is through an alteration in Ca\(^{++}\) binding to mitochondrial and, perhaps, other membrane systems, including the plasma membrane and the endoplasmic reticulum (264).

**Other Hypoglycaemic Agents.**

There are several other hypoglycaemic agents, which are worthy of a brief mention in this introduction because of their different modes of action. These include the indole and quinoline derivatives, dichloroacetate, nicotinate, hypoglycin and pent-4-enoate, and diphenyleneiodonium.

**Indole And Quinoline Derivatives.**

Of the indole and quinoline derivatives, both quinolinic acid and 3-mercaptopicolinate have been extensively investigated. These agents are believed to exert their hypoglycaemic effects through inhibiting the enzyme phosphoenolpyruvate carboxykinase (PEPCK) and thus inhibiting gluconeogenesis (275-278). Both quinolinic acid (274) and 3-mercaptopicolinate (275) have been shown to have hypoglycaemic effects in starved and diabetic animals, but to have little effect on blood glucose levels in normal, fed animals; this is an indication that they inhibit gluconeogenesis, since the process operates minimally in the post-absorptive state.

Ditullio and coworkers (274) showed that 3-mercaptopicolinate inhibited gluconeogenesis in perfused livers from lactate, pyruvate and alanine, but had no effect on the rate of gluconeogenesis from fructose or dihydroxyacetone. These results indicated that the site of inhibition was probably at the rate-limiting step controlled by pyruvate carboxylase, PEPCK and pyruvate kinase. A cross-over plot, calculated from the metabolite concentrations in freeze-clamped perfused livers, indicated the phosphoenolpyruvate carboxykinase reaction as a possible site of inhibition (276, 277). Similar results have been obtained with quinolinic acid (275, 278).

A possible mechanism for the inhibition of phosphoenolpyruvate...
carboxykinase has been proposed (279) based on the ability of these compounds to bind to Fe$$^{++}$$ (or Mn$$^{++}$$), which are in turn bound to and activate PEPCK. The presence of the agent at the metal-ion binding site interferes in some way with the catalytic site of PEPCK.

Dichloroacetate.

Dichloroacetate has been shown to be hypoglycaemic in normal, fasted rats (280, 281) and in alloxan-diabetic rats (282-286), but not in normal, fed rats (282-284). It increases glucose oxidation and inhibits fatty acid oxidation in diaphragm muscle of diabetic rats (283, 284) and increases glucose, lactate and pyruvate extraction but decreases fatty acid oxidation in perfused heart (287, 288). Whitehouse and coworkers (281, 288) have shown that dichloroacetate activates pyruvate dehydrogenase (PDH), probably due to inhibition of pyruvate dehydrogenase kinase, and have proposed this as the mechanism of action of dichloroacetate.

Blackshear and coworkers (286) have shown that dichloroacetate causes a decrease in the accumulation of lactate, pyruvate and alanine in the blood of functionally hepatectomized diabetic animals. These findings are consistent with activation of extrahepatic PDH and support the view that dichloroacetate causes hypoglycaemia by decreasing the net release of gluconeogenic precursors from extrahepatic tissues (280, 281).

Nicotinate.

Nicotinate has been shown to be hypoglycaemic in fasted, normal and alloxan diabetic rats (289-292), in mice (293) and during infusions into man (294-296). This lowering of blood sugar is combined with an increased respiratory quotient indicating an increased oxidation of carbohydrates (297, 298). However, nicotinate has also been shown to have no effect on blood glucose levels in man (299) and to actually increase them (300, 304). The diversity of these results seems to come from the different experimental conditions used, but it does seem likely that nicotinate affects glucose tolerance by more than one mechanism.

Dzedin et al (305) have suggested that the stimulatory effect of nicotinate on blood glucose levels is at least partially dependant on
Nicotinate is believed to lower blood glucose levels by virtue of its antilipolytic activity (306-312). Randle and coworkers (313,314) have demonstrated that the increased oxidation of fatty acids which results from a rise of plasma free fatty acids brings about the inhibition of glucose metabolism (they showed an inhibitory effect of free fatty acids and ketones on the uptake and phosphorylation of glucose in isolated muscle preparations). The increase in free fatty acid oxidation brings about a rise in acetyl CoA formation and an inhibition of several enzymes of the glucose metabolic pathways.

Mechanisms have been proposed for the inhibitory effects of diabetes and of free fatty acid oxidation on pyruvate dehydrogenase, phosphofructokinase and hexokinase (315-320). Fatty acids and diabetes have been shown to increase the ratio of acetyl CoA to CoA in the perfused heart (321) and such an increase has been shown to inhibit the oxidation of pyruvate to acetyl CoA by pyruvate dehydrogenase (318,322). The increased acetyl CoA/CoA ratio may also lead to increased citrate formation through citrate synthase. Citrate is an inhibitor of phosphofructokinase and elevated levels of citrate have been shown to be induced by fatty acid oxidation and by diabetes (319,320,323). The inhibitory effects of free fatty acids and diabetes on glucose phosphorylation have been attributed to the inhibition of hexokinase by glucose-6-phosphate. The concentration of glucose-6-phosphate is increased in muscle as a result of inhibition of phosphofructokinase (314). Thus, an increase in the acetyl CoA/CoA ratio can cause an inhibition of glucose phosphorylation, glycolysis and pyruvate oxidation. This mechanism could explain both the decreased glucose utilization following the rise of plasma free fatty acids by a fat infusion and the improved glucose tolerance following the lowering of plasma free fatty acids during perfusion of an antilipolytic agent, such as nicotinate.
Hypoglycaemia is caused by hypoglycin (L-2-amino-3-methylenecyclopropylpropionic acid) the toxic principle of the Jamaican ackee fruit, Blighia sapida (324-326). The structurally related compound, pent-4-enoate has also been shown to cause hypoglycaemia in many species. Both compounds interfere with β-oxidation and gluconeogenesis in a variety of *in vitro* systems (327-338). It has been suggested that hypoglycin and pent-4-enoate inhibit gluconeogenesis secondary to their inhibition of fatty acid oxidation, such that glucose is the only major metabolic fuel and when glycogen is exhausted, hypoglycaemia ensues (339). Plasma free fatty acids have been shown to be elevated after hypoglycin (340) or pent-4-enoate (341) administration to rats. Also hypoglycin poisoning is associated with fatty livers (342), when presumably free fatty acids are supplied from adipose tissue stores in excess of the amounts that are oxidized to carbon dioxide and ketone bodies, and are therefore converted to triglycerides. Methylenecyclopropylacetate CoA (methylenecyclopropylacetate is the active metabolite of hypoglycin) inhibits the maximum rate of oxidative generation of acetyl CoA from palmitoyl-carnitine by isolated rat liver mitochondria (331).

Billington and coworkers (338,343) have proposed that the accumulation of high concentrations of several acyl CoA esters in the mitochondrial matrix will interfere with gluconeogenesis by inhibiting the activation of pyruvate carboxylase by acetyl CoA. In support of this proposal, Williamson and coworkers (336) and Toews and coworkers (344) have produced evidence with crossover plots of liver metabolite levels in the presence of pent-4-enoate that showed pyruvate carboxylase was inhibited.

However, there are other effects of these agents whose contribution to their hypoglycaemic action is not yet defined. For example, it has recently been demonstrated that the administration of hypoglycin to rats can severely inhibit both gluconeogenesis and glucose utilization (345, 346). There is, however, no quantitative information about the effects of hypoglycin or pent-4-enoate on fatty acid oxidation *in vivo*, nor on the
effects of pent-4-enoate on glucose metabolism in vivo (346). Both hypoglycin and pent-4-enoate also cause profound hypothermia in small animals, which will decrease the absolute rates of metabolism compared with control animals (338). However, there are no measurements of the effects of hypoglycin or pent-4-enoate on oxygen consumption in animals maintained in thermoneutral conditions, so that the contribution of the hypothermia to their metabolic effects is not clear.

Diphenyleneiodonium.

Diphenyleneiodonium causes extreme and irreversible hypoglycaemia in several animal species (347, 348) associated with a potent inhibition of gluconeogenesis (348, 349). High doses of diphenyleneiodonium were also shown to cause a stimulation of whole body glucose utilization and lactate production, but this was not apparent with lower doses where hypoglycaemia was still observed (348). Diphenyleneiodonium has been shown to decrease the $\frac{[ATP]}{[ADP][HPO_4^{2-}]}$ ratio and to decrease the $\frac{[NAD^+]}{[NADH]}$ ratio (348, 349). These results suggest that diphenyleneiodonium inhibits gluconeogenesis by virtue of its ability to inhibit mitochondrial oxidation. Holland and Sherratt (350-352) showed that diphenyleneiodonium inhibits State 3 and dinitrophenol-stimulated oxidation of succinate and glutamate by isolated mitochondria. They proposed that these inhibitory effects of diphenyleneiodonium were a consequence of the compulsory exchange of chloride ions for hydroxyl ions across the inner mitochondrial membrane, which they found diphenyleneiodonium caused in their experiments (349). However, their in vitro experiments required high concentrations of diphenyleneiodonium and Holland and coworkers (348) were unable to show this effect in latter in vivo work. It seems likely that diphenyleneiodonium has a direct action on mitochondrial metabolism as it causes marked inhibition of glutamate-malate oxidation in mitochondria (348). Further evidence comes from the fact that diphenyleneiodonium inhibits NADH oxidation in submitochondrial particles and the oxidation of NADH-linked substrates added in vitro to intact mitochondria (348).
Concluding Remarks.

Thus, there is a wide variety of mechanisms by which a hypoglycaemic agent can lower blood glucose levels. Very little was known about the hypoglycaemic agent, Wy 23675, when the present study was started, so that initially in vivo comparisons were made with other hypoglycaemic agents. These studies were followed by in vitro studies with hemidiaphragms, isolated adipocytes and isolated hepatocytes and, finally, a measurement of liver metabolite levels. The results of this work are reported in this thesis.
Introduction
References

References


References

Introduction References.

Introduction References.


Introduction References.

154. Loubatières, A. Arch. Int. Physiol. 54, 174-177, 1946.
Introduction References.


Introduction

References:


Introduction References.

256. Elkeles, R. S. Meeting of Medical and Scientific Section of The British Diabetic Association, York, April, 1972.
Introduction References.

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CHAPTER 1.
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The figures in this section illustrate the effects of Wy 23675 on blood glucose and plasma insulin levels; and compares these effects with those of the standard oral hypoglycaemic agents, tolbutamide (a sulphonylurea) and phenformin (a biguanide), used in this study. The results illustrate the following effects of these agents:

1. Oral or intravenous administration of Wy 23675 and tolbutamide results in hypoglycaemia in the normal rat; phenformin has no effect.

2. Oral administration of Wy 23675 and tolbutamide elevates plasma insulin levels in the normal rat; phenformin has no effect.

3. Intravenous administration of Wy 23675 in low doses, which are still markedly hypoglycaemic, has no effect on plasma insulin levels; intravenous administration of tolbutamide elevates plasma insulin levels.

4. Inhibition of insulin release by diazoxide does not markedly effect the hypoglycaemic action of Wy 23675, while it inhibits that of tolbutamide.

5. Wy 23675, tolbutamide and phenformin are hypoglycaemic in the glucose-loaded rat.

6. Tolbutamide further elevates glucose-stimulated plasma insulin levels in the glucose-loaded rat, while neither phenformin nor Wy 23675 have any further effect.

7. Thus, these results indicate that Wy 23675, tolbutamide and phenformin have dissimilar modes of hypoglycaemic action.
EARLY STUDIES ON THE HYPOGLYCAEMIC AGENT WY 23675.

The hypoglycaemic agent, Wy 23675, has been under investigation at Wyeth Laboratories, Taplow as a potential antidiabetic agent since 1973, following the discovery of its hypoglycaemic action. During 1975 it was appreciated that the toxicity of Wy 23675 precluded its development as a therapeutic agent for clinical use; however, its novel chemical structure and possible novel mode of action have made it worthy of further investigation. Furthermore, it is related in structure to other compounds developed by Wyeth Laboratories, which have shown a similar insulin-independent hypoglycaemic activity, but without the poor therapeutic ratio exhibited by Wy 23675. Examples of these compounds include mazindol and ciclazindol (1-4).

In structure Wy 23675 is chemically dissimilar to existing drugs, which are in clinical use as hypoglycaemic agents (Figure 1(i)). The most commonly used groups of orally-administered hypoglycaemic agents are the sulphonylureas and the biguanides. Mechanistically, the former are thought to act primarily by an action on the pancreatic β-cells (although studies with chronic administration of sulphonylureas have indicated extra-pancreatic effects such as direct action on the liver or peripheral potentiation of the glucose-lowering effectiveness of insulin) and the latter through an action other than stimulation of insulin release from the pancreatic β-cells (5). It was important to consider to what extent the mechanism of Wy 23675 could be attributed to an insulin-independent action.

Chemistry.

Wy 23675 was originally developed as a structural analog of another Wyeth compound (Wy 23117) being investigated for its antidepressant potential. This series of compounds had previously shown marked hypoglycaemic activity; and in Wy 23675 this was very marked, which led to its being considered as a potential antidiabetic agent.
Methods.

1. General.

Rats used throughout the study, unless otherwise stated, were Charles River CD strain, weights 180-220 gms. They were fed on rat pellets (Oxoids 413) and kept in cages of 4 in rooms at 22°C with a 12 hour light/dark cycle. Drugs administered by the oral route were made up in 0.5% hydroxypropylmethyl cellulose in 0.9% saline (HPMC) vehicle in a dose volume of 1 ml/100 g. body weight. Drugs administered by the intravenous route were made up in 0.9% saline in a dose volume of 0.1 ml/100 g. body weight; all intravenous injections were made into the tail vein.


Wy 23675 was synthesized by Wyeth Laboratories, Maidenhead, Berks. Tolbutamide was supplied by Hoechst Pharmaceuticals, Hounslow, Middx. Phenformin was supplied by Winthrop Laboratories, Surbiton, Surrey. Diazoxide was supplied by Allen & Hanburys Ltd, Ware, Herts.


Blood samples were taken throughout the study by cutting the tip off the tail of unanaesthetized rats, held in Perspex cylinders with a groove cut down the top to allow the tail to pass through, and gently massaging it. 0.5 mls of blood was collected into fluoride pots for determinations of blood glucose by the ferricyanide method - Technicon Autoanalyser Method N-16b. (Blood glucose determinations were made by the staff of the Clinical Chemistry Section at Wyeth Laboratories, Taplow).

4. Plasma insulin determinations.

Plasma insulin levels were estimated by a competitive binding radioimmunoassay method on 0.3 mls of blood samples obtained from the tails of rats as above and centrifuged in a Beckman microfuge for 4 minutes. The Lepetit radioimmunoassay kit for insulin, which was used, relies upon the
reaction between the insulin to be measured and a standard amount of antibody, to form an antibody-insulin complex. The concentration of antibody has been selected to restrict the amount of insulin allowed to combine, so that following reaction, some fraction of the insulin originally present remains in the uncombined or 'free' form. Therefore, as the total amount of insulin present in the system increases, so the 'free' fraction will increase, and conversely the proportion of insulin 'bound' to the antibody will decrease.

A tracer amount of radioactive-labelled insulin introduced into the reaction mixture reflects the distribution of unlabelled and labelled insulin between the free and bound forms. The distribution of radioactive insulin following the reaction thus provides an easy measurement, which indicates the amount of unlabelled insulin in the system.

A standard response curve or calibration curve is plotted showing the counts recorded from the unbound insulin fraction against the known concentrations of unlabelled insulin introduced into the standard incubation tubes. Insulin concentrations in blood samples are estimated by comparison with the standard response curve.

The Lepetit kit differs from others principally in the means employed to isolate the free from the antibody-bound labelled insulin following reaction. The Lepetit kit relies upon the adsorption of free insulin onto activated charcoal, the antibody-bound radioactivity remaining in the supernatant solution. In contrast a radioimmunoassay kit for insulin from the Radiochemical Centre, Amersham involves a double antibody technique, where separation of free from bound insulin fractions is by adsorption of the insulin-bound-antibody complex onto a second antibody forming an insoluble complex. The free insulin remains in the supernatant which is discarded, and the bound complex is counted for $^{125}$I-insulin. The overall time for the assay using the Radiochemical Centre kit is longer, as longer incubation period is required between the insulin and insulin-antibody.

**Effects on Blood Glucose and Plasma Insulin Levels.**

My 23675 has been shown to have oral hypoglycaemic activity in the
24-hr fasted, fed, glucose-loaded and alloxan-diabetic rat (Table 1) and in the normoglycaemic mouse, dog and Patas monkey (Wyeth Laboratories, unpublished results). The results for Wy 23675 in the rat are compared with results from similar experiments with tolbutamide and phenformin in Table 1. Tolbutamide had hypoglycaemic effects in all situations where it was able to stimulate insulin release (ie: 24-hr fasted, fed and glucose-loaded rats), but not where insulin release could not be stimulated (ie: the alloxan-diabetic rat). Phenformin lowered blood glucose levels in the fasted, glucose-loaded and alloxan-diabetic rat, but not in the normal, fed rat.

Figure 1(ii) illustrates the oral hypoglycaemic activity of Wy 23675 as compared with that of the sulphonylurea, tolbutamide, in normoglycaemic, fed rats. The hypoglycaemic effect of Wy 23675 occurs over a narrower dose range than that of tolbutamide, first becoming apparent at about 0.04 mmol/kg p.o. and almost maximal by 0.4 mmol/kg p.o. Tolbutamide appears to be less potent than Wy 23675 at low doses, but its maximal hypoglycaemic activity is markedly greater than that achieved with Wy 23675. The biguanide, phenformin, showed no hypoglycaemic effects in the normoglycaemic fed rat at doses up to 0.8 mmol/kg p.o. (results at Wyeth Laboratories, not shown).

Wy 23675 administered by the oral route to normoglycaemic fed rats stimulates insulin release (Fig. 1(iii)), whereas given intravenously at lower doses, it has no effect on plasma insulin levels, but retains its hypoglycaemic action (Fig. 1(iv)). When animals were pretreated with diazoxide, in order to suppress insulin release from the pancreas(7,8), the oral administration of Wy 23675 still provoked a hypoglycaemic response despite the lack of stimulation of insulin release (Fig. 1(v)). In glucose-loaded rats, the increase in plasma insulin concentration is not further stimulated by the simultaneous oral administration of Wy 23675 until after 40 minutes, whereas the increase in blood glucose levels is considerably diminished by Wy 23675 by 20 minutes (Fig. 1(vi)). However, it is possible that part of the early hypoglycaemic action of
Viy 23675 may be attributed, in this case, to a potentiation of the action of insulin already released by the glucose-load. At 60 minutes after treatment, the difference in blood glucose concentration between the rats treated with glucose alone and glucose plus Wy 23675 may be related in part to the persistence of hyperinsulinaemia in the Wy 23675-treated rats. However, taken together Figures 1(iv), 1(v), and 1(vi) provide good evidence for an insulin-independent hypoglycaemic action of Wy 23675.

The actions of Wy 23675 differ from those of both tolbutamide and phenformin. The hypoglycaemic action of tolbutamide is accompanied by increases in plasma insulin levels for both oral (Fig. 1(vii).) and intravenous administration (results not shown, but similar to Fig. 1(vii).) When diazoxide blockage of insulin release is complete, tolbutamide has no effect on blood glucose levels (Fig. 1(viii).). Progressive increases in doses of tolbutamide, which overcome the diazoxide-blockage of insulin release, result in a progressively greater hypoglycaemic effect. In the glucose-loaded rat, tolbutamide lowers raised blood glucose levels in relation to its ability to stimulate plasma insulin levels further (Fig. 1(ix).); this is unlike Wy 23675 (Fig. 1(vi).).

Phenformin, on the other hand, does not markedly affect plasma insulin levels and, unlike Wy 23675 or tolbutamide, has no effect on blood glucose concentrations in the normoglycaemic rat (Fig. 1(x).). In glucose-loaded rats, phenformin lowers blood glucose levels without affecting plasma insulin levels (Fig. 1(xi).).

Effects on Growth Hormone and Glucagon Levels.

The possibility that the insulin-independent hypoglycaemic action of Wy 23675 is mediated by other hormones affecting carbohydrate metabolism has been considered. No effects of Wy 23675 have been measured on plasma growth hormone levels at doses at which it is hypoglycaemic; and hypophysectomy has been shown to have no effect on the hypoglycaemic action of Wy 23675 (results not shown).

Tests for Glycosuric Effects.

The possibility that Wy 23675 lowered blood glucose levels by increasing the loss of glucose in urine has been investigated. Twenty-four
hour fasted rats were given a glucose-load combined with a hypoglycaemic
dose of Wy 23675 and left in rat metabolism cages with free access to
water for 5 hours, during which the urine was collected. The group given
Wy 23675 were not significantly different to the control group with
respect to either total urine volume or urine glucose produced.

Toxicity Studies.

The possibility that the hypoglycaemic action of Wy 23675 could be
an indirect consequence of general toxicity has also been considered.
However, rats dosed with Wy 23675 (0.2 mmol/kg p.o.) twice-a-day for 31
days developed no tolerance to its hypoglycaemic action and no effects
were seen on the mean rat growth rate or on histological examination of
selected tissues (adrenals, testes, lungs, heart, kidney or liver) or on
serum chemistry (albumin, globulin, total protein, lipids, cholesterol,
alkaline phosphatase, aspartate transaminase, alanine transaminase, sodium
or potassium). (results not shown).

Pharmacokinetic Studies.

Studies of the plasma kinetics of Wy 23675 in Patas monkeys have
revealed that peak plasma concentrations of Wy 23675 are reached within
2 hours of an oral dose of the drug. Following intravenous administration
of Wy 23675, plasma levels remained fairly steady over a ten hour period.
Furthermore, it was found that plasma levels after intravenous dosage
were similar to oral levels after correcting for the different dosages.

An in situ gut absorption study was considered to offer a more direct
means of establishing that the drug was rapidly absorbed. An isolated
intestinal loop cannulated at both ends in an anaesthetized monkey
showed that within 10 minutes of introducing Wy 23675 into the isolated
gut section, the concentration had fallen by 90%.

Thus, Wy 23675 is rapidly absorbed and only minimally, if at all,
metabolized during its passage through the gut. Following an oral dose
of 0.2 mmol/kg, plasma concentrations of Wy 23675 were 0.12 mM and 0.16 mM
in two different Patas monkeys.
COMPARISON OF THE EFFECT OF Wy 23675 WITH THAT OF Tolbutamide AND Phenformin ON BLOOD GLUCOSE AND PLASMA INSULIN LEVELS IN NORMAL RATS.

Figure No. | Title
---|---
1(i) | Structure and Physical Properties of Wy 23675.
1(ii) | Effects of Wy 23675 and Tolbutamide on Blood Glucose Levels Two Hours after Oral Dosing.
1(iii) | Effect of Oral Administration of Wy 23675 on Peripheral Blood Glucose and Plasma Insulin Levels.
1(iv) | Effect of Intravenous Administration of Wy 23675 on Peripheral Blood Glucose and Plasma Insulin Levels.
1(v) | Effect of Oral Administration of Wy 23675 on Peripheral Blood Glucose and Plasma Insulin Levels in Diazoxide-Treated Rats.
1(vi) | Effect of Oral Administration of Wy 23675 on Glucose-Stimulated Insulin Release.
1(vii) | Effect of Oral Administration of Tolbutamide on Peripheral Blood Glucose and Plasma Insulin Levels.
1(viii) | Effect of Oral Administration of Tolbutamide on Peripheral Blood Glucose and Plasma Insulin Levels in Diazoxide-Treated Rats.
1(ix) | Effect of Oral Administration of Tolbutamide on Glucose-Stimulated Insulin Release.
1(x) | Effect of Oral Administration of Phenformin on Peripheral Blood Glucose and Plasma Insulin Levels.
EFFECTS OF TOLBUTAMIDE, PHENFORMIN AND WY 23675 ON BLOOD GLUCOSE LEVELS IN 24-HR. FASTED, FED, GLUCOSE-LOADED AND ALLOXAN-DIABETIC RATS.

Groups of 6 female rats (180-220gm) were dosed orally with the drugs (0.2mmol/kg) and blood samples were taken for glucose measurement at 0, 1, 2 and 3 hrs afterwards. Fasted rats were deprived of food for 24 hrs prior to the experiment. Glucose-loaded rats were simultaneously dosed orally with glucose (10 mmol/kg) and with drugs. Alloxan-diabetic rats were treated with alloxan (0.7mmol/kg i.p), following a 24-hr fast, 7 days prior to the experiment when animals were found to be frankly diabetic (as judged by glucosuria). Statistical significance was determined by Student's t-Test: * p<0.05; ** p<0.01; *** p<0.001

Zero time control blood glucose levels were:
- 24-hr. fasted: 3.03 mmol/l.
- Fed: 4.24 mmol/l.
- Glucose-loaded: 5.20 mmol/l. (1 hr after glucose-load).
- Alloxan-diabetic: 19.41 mmol/l.

<table>
<thead>
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<th>Change in Blood Glucose Levels (mmol/l.) from Zero Time Point Relative to Control at the Following Times:</th>
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<tr>
<td></td>
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<td><strong>24-hr. Fasted Rats.</strong></td>
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<td>Tolbutamide</td>
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<tr>
<td>Phenformin</td>
<td>-0.83**</td>
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<tr>
<td>Wy 23675</td>
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<tr>
<td>Phenformin</td>
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<td>Wy 23675</td>
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<td><strong>Glucose-loaded Rats.</strong></td>
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<tr>
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<td><strong>Alloxan-diabetic Rats.</strong></td>
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<tr>
<td>Phenformin</td>
<td>-5.41*</td>
</tr>
<tr>
<td>Wy 23675</td>
<td>-9.72*</td>
</tr>
</tbody>
</table>

\[45\]
Diphenyl-(1,4,5,6-tetrahydro-2-pyrimidyl)-methanol

Empirical formula: $\text{C}_{17}\text{H}_{18}\text{N}_2\text{O}$

Molecular weight: 266

Melting point: 115-118°C

Chemical purity: 99%+ on TLC.

Wy 23675 is a white crystalline solid, virtually odourless with a slightly bitter taste.

Phenformin

\[
\text{NH} \quad \text{NH} \\
\text{CH}_2\text{CH}_2\text{NHC-NH-C-NH}_2 
\]

HCl

Tolbutamide

\[
\text{SO}_2\text{NHCONHCH}_2\text{CH}_2\text{CH}_2\text{CH}_3
\]
Figure 1(ii)

EFFECTS OF Wy 23675 AND TOLBUTAMIDE ON BLOOD GLUCOSE LEVELS TWO HOURS
AFTER ORAL DOSING.

Normoglycaemic fed female rats were dosed orally with Wy 23675 or
tolbutamide as described in Methods. Blood samples were withdrawn
immediately before and 2 hrs after dosing and the glucose concentration
measured. The results below summarize three experiments done at the
same time on consecutive days. Each point is the Mean (± SEM) of 6
observations and represents the % change in blood glucose concentration
2 hours after oral dosing with Wy 23675 (○) or tolbutamide (▽) from the
value before dosing and relative to a similar value for the control
group dosed with vehicle. The blood glucose concentration of control
animals was 5.01 (±0.17) mmol of glucose/litre of blood prior to
dosing and 5.45 (±0.11) mmol/l. at the 2 hr time-point.
Figure 1(ii)

Oral Dose of Drug (mmol/kg) log. scale

% Change in Blood Glucose Levels Relative to Control

Wy 23675

Tolbutamide
Normoglycaemic fed female rats were dosed orally with Wy 23675 (0.2 mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 mins. Each point is the Mean (± SEM) of 6 observations and shows the difference in concentrations of blood glucose or plasma insulin from that at zero time (time of dosing). Blood glucose and plasma insulin concentrations at zero time were 4.95 (±0.11) mmol/l & 32 (±5) µUnits/ml, respectively, for the control group.
Figure 1(iii)

- Change in Blood Glucose (mmol/L)
- Change in Plasma Insulin Levels (μUnits/ml)

- Time after Dosing (minutes)
- Wy 25675
- Control
Figure 1(iv)
EFFECT OF INTRAVENOUS ADMINISTRATION OF WY 23675 ON PERIPHERAL BLOOD GLUCOSE
AND PLASMA INSULIN CONCENTRATIONS.

Normoglycaemic fed female rats were injected intravenously via the tail vein with Wy 23675 (0.8mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 mins. Each point is the Mean (± SEM) of 6 observations and shows the difference in concentrations of blood glucose or plasma insulin from that at zero time (time of injection). Blood glucose and plasma insulin levels at zero time were 5.42(±0.17)mmol/l + 29 (± 4) µUnits/ml, respectively, for the control group.
EFFECT OF ORAL ADMINISTRATION OF WY 23675 ON PERIPHERAL BLOOD GLUCOSE AND
PLASMA INSULIN CONCENTRATION IN DIAZoxide-TREATED RATS.

Normoglycaemic fed female rats were pretreated with oral administration of diazoxide (1.6 mmol/kg) and then 1 hr later were given WY 23675 (0.2 mmol/kg) by the oral route. Blood samples were taken for glucose and insulin measurement at time intervals up to 60 min after dosing with WY 23675. Each point is the Mean (± SEM) of 6 observations and shows the difference in concentrations of blood glucose or plasma insulin from that at zero time (time of WY 23675 administration). Blood glucose and plasma insulin levels at zero time were 7.15 (± 0.61) mmol/l and 17 (± 3) µUnits/ml, respectively, for the control group.
Normoglycaemic fed female rats were dosed orally simultaneously with glucose (10 mmol/kg) and with Wy 23675 (0.2 mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 min. Each time point is the Mean (± SEM) of 6 observations and shows the differences in concentrations of blood glucose or plasma insulin from that at zero time (time of dosing). Blood glucose and plasma insulin levels at zero time were 5.23 (±0.17) mmol/l and 36 (±6) µUnits/ml, respectively, for the control group.
Figure 1(vi)

Change in Blood Glucose (mmol/l.)

<table>
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<th>Time after Dosing (minutes)</th>
<th>Change in Plasma Insulin Levels (µUnits/ml)</th>
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</thead>
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<td>Wy 23675</td>
</tr>
<tr>
<td>40</td>
<td></td>
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<tr>
<td>60</td>
<td></td>
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</tbody>
</table>

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Figure 1(vii)

EFFECT OF ORAL ADMINISTRATION OF TOLBUTAMIDE ON PERIPHERAL BLOOD GLUCOSE AND PLASMA INSULIN CONCENTRATIONS.

Normoglycaemic fed female rats were dosed orally with tolbutamide (0.2 mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 min. Each point is the Mean (± SEM) of 6 observations and shows the difference in concentrations of blood glucose or plasma insulin from that at zero time (time of dosing). Blood glucose and plasma insulin concentrations at zero time were 5.12 (±0.11) mmol/l and 31 (±3) μUnits/ml, respectively, for the control group.
Figure 1(viii)

EFFECT OF ORAL ADMINISTRATION OF TOLBUTAMIDE ON PERIPHERAL BLOOD GLUCOSE AND PLASMA INSULIN CONCENTRATION IN DIAZOXIDE-TREATED RATS.

Normoglycaemic fed female rats were pretreated with oral administration of diazoxide(1.6 mmol/kg) and then 1 hr later were given tolbutamide (0.025, 0.05, 0.2 mmol/kg) by the oral route. Blood samples were taken for glucose and insulin measurement at time intervals up to 60 min after dosing with tolbutamide. Each point is the Mean (± SEM) of 6 observations and shows the difference in concentration of blood glucose or plasma insulin from that at zero time (time of tolbutamide administration). Blood glucose and plasma insulin levels at zero time were 7.81 (±0.50) mmol/l, and 9 (±4) μUnits/ml, respectively, for the control group.
Figure 1(ix)
EFFECT OF ORAL ADMINISTRATION OF TOLBUTAMIDE ON GLUCOSE-STIMULATED INSULIN RELEASE.

Normoglycaemic fed female rats were dosed orally simultaneously with glucose (10 mmol/kg) and with tolbutamide (0.2 mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 min. Each time point is the Mean (± SEM) of 6 observations and shows the differences in concentrations of blood glucose and plasma insulin from that at zero time (time of dosing). Blood glucose and plasma insulin levels at zero time were 5.17 (±0.17) mmol/l and 28 (±5) μUnits/ml, respectively, for the control group.
Figure 1(x)

EFFECT OF ORAL ADMINISTRATION OF PHENFORMIN ON PERIPHERAL BLOOD GLUCOSE AND PLASMA INSULIN CONCENTRATIONS.

Normoglycaemic fed female rats were dosed orally with phenformin (0.2 mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 min. Each point is the Mean (± SEM) of 6 observations and shows the differences in concentrations of blood glucose or plasma insulin from that at zero time (time of dosing). Blood glucose and plasma insulin concentrations at zero time were 5.28 (±0.22) mmol/l, and 31 (±4) μUnits/ml, respectively, for the control group.
Normoglycaemic fed female rats were dosed orally simultaneously with glucose (10 mmol/kg) and with phenformin (2 mmol/kg) and blood samples were taken for glucose and insulin measurements at time intervals up to 60 min. Each time point is the Mean (± SEM) of 6 observations and shows the differences in concentrations of blood glucose or plasma insulin from that at zero time (time of dosing). Blood glucose and plasma insulin levels at zero time were 4.90 (±0.17) mmol/l and 29.15 μUnits/ml, respectively, for the control group.
Wy 23675 is a hypoglycaemic agent of novel structure and with a mode of action apparently different from those of the two major groups of oral hypoglycaemic agents, the sulphonylureas and the biguanides, as represented by tolbutamide and phenformin, respectively. It is apparent from the results on blood glucose and plasma insulin levels, that Wy 23675 has a hypoglycaemic action which is independent of, although it can stimulate, insulin release.

It is interesting that Wy 23675 causes the release of insulin when administered by the oral route, while in low doses, it does not affect plasma insulin levels by the intravenous route, although it is still hypoglycaemic. These results suggest that Wy 23675 does not have a direct effect on the pancreas to stimulate insulin release. There are three possible ways by which Wy 23675 could stimulate insulin release when administered by the oral route but not by the intravenous route.

First, a metabolite of Wy 23675 could be formed during its passage through the gut and it is this metabolite which stimulates insulin release. This suggestion is unlikely in view of the pharmacokinetic studies with Wy 23675 described in this chapter.

Secondly, Wy 23675 may cause a neurogenic effect on insulin release, mediated through the vagus nerve. Histological studies have shown that fibres from the right vagus nerve innervate the pancreas in man, dog, cat and rabbit (17). Plexuses of such fibres surround the islets, and filaments from these peri-insular networks penetrate the islets and follow the vessels between the cells. In 1927 LaBarre (18), using the method of pancreatoduodenal-jugular anastomosis in the dog, demonstrated a fall in blood glucose of the recipient following an electrical stimulation of the right vagus nerve of the donor. Frohman and coworkers (9) have shown that stimulation of the right and left vagus leads to a rapid rise in serum insulin levels and that atropine inhibits this effect. They have also shown that, following vagotomy, a fall in portal vein insulin levels occurs, but that the glucose-mediated insulin release and net glucose utilization are not affected by vagotomy. Isolated tissue work at Wyeth
although it does cause some potentiation of acetyl choline. Thus, the possibility of Wy 23675 stimulating insulin release through vagal effects cannot be eliminated. However, the pattern of insulin release caused by Wy 23675 is different from that caused by vagal stimulation. Frohman et al (9) have shown that, in the dog, vagal stimulation results in a prompt but short-lived peaking of plasma insulin release before 5 minutes despite the persistence of stimulation. They conclude that only a portion of releasable insulin appears to be under vagal control. In contrast, Wy 23675 results in a gradual and sustained release of insulin over a period in excess of one hour, so it seems unlikely that there is a vagal involvement in Wy 23675 stimulation of insulin release.

The third possibility is that Wy 23675 could, when given orally, stimulate the release from the gut of an insulin-releasing factor. The existence of such a factor was suggested at the beginning of this century, shortly after the discovery of secretin, when acid extracts of the duodenum were used in the treatment of diabetes mellitus (19). It was suggested that crude secretin contained two active principles: 'incretin', stimulating the internal secretion of the pancreas, and 'excretin', stimulating the exocrine pancreas (20,21). More recently, it has been shown that serum insulin levels increased much more after oral administration than after intravenous injection of the same amount of glucose, despite a smaller increase in blood glucose levels (22,23). Similarly, intraduodenal administration of amino acids has been shown to induce a greater rise in serum insulin than intravenous infusion despite lower serum levels for the individual amino acids in the former (24). Many gastrointestinal factors (secretin, gastrin, gastric inhibitory polypeptide-GIP, vasoactive intestinal polypeptide-VIP) have been shown to be capable of stimulating insulin (14,25-29), but the enteric factor responsible for greater insulin release after oral glucose ingestion or intraduodenal infusion compared with an intravenous glucose infusion has yet to be identified. Of the different candidate hormones, GIP fulfills all the criteria necessary for the role
glucose and fat (10-13) and to be insulinotropic in the presence of hyperglycaemia (13-16). The possibility that Wy 23675 stimulates the release of an insulin-release-stimulating factor from the gut, such as GIP, has not been looked into.

Thus, Wy 23675 appears to have a hypoglycaemic activity, which has an insulin-independent component, is unrelated to growth hormone levels, and independent of any toxic effect.
18. LaBarre, J. Compte Rend. Soc. de Biol. 96, 193, 1927.


THE EFFECTS OF INSULIN, TOLBUTAMIDE, PHENFORMIN AND WY 23675 ON GLUCOSE UTILIZATION 'IN VIVO'.

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Chapter 2: Summary

The tables in this section summarize the results of experiments designed to illustrate the 'in vivo' effects of selected hypoglycaemic agents on glucose utilization as distinct from glucose production. The results illustrate the following effects of these agents:

1. Insulin increased the rate of glucose utilization and lowered blood glucose levels in both normal and alloxan-diabetic rats.

2. In the normal rat, oral administration of tolbutamide and Wy 23675 elevated plasma insulin levels, stimulated glucose utilization and lowered blood glucose levels; phenformin had no effect.

3. Intravenous administration of Wy 23675 in low doses in the normal rat had no effect on plasma insulin levels, but stimulated glucose utilization and lowered blood glucose levels while intravenous administration of tolbutamide stimulated both plasma insulin levels and glucose utilization, and lowered blood glucose levels.

4. Inhibition of insulin release by diazoxide did not markedly affect Wy 23675-stimulation of glucose utilization or lowering of blood glucose levels, but inhibited these effects of tolbutamide.

5. In the alloxan-diabetic, Wy 23675 and phenformin stimulated glucose utilization and lowered blood glucose levels; tolbutamide had no effect. None of these agents had any measurable effect on plasma insulin levels.

6. In all cases, the effects of these agents on glucose utilization paralleled their effectiveness in causing hypoglycaemia, indicating that glucose utilization is part of the mechanism by which these agents induce a fall in blood glucose levels.
Radiolabelled glucose has been used both to measure the rate of glucose utilization and the disposal of glucose carbon to selected tissues and end-products (1-29). These studies used (U-$^{14}$C)-glucose, which meant that measurements of radiolabelled glucose in the blood included not only the original (U-$^{14}$C)-glucose injected, but also newly-synthesized glucose labelled with $^{14}$C due to substrate recycling. Baker et al took this into account by including recycling from a non-glucose compartment in their 3-pool model (3), but this technique requires a careful and fairly complex analysis of the results. Vrba et al (8-12, 14-16) did not have this problem as they were analyzing end products which represented large pools in the body, so that the tracer became sufficiently diluted that negligible loss of it accompanied the outflow of natural material from the pool. In this way the pool became an effective 'sink' and a plateau value of tracer was reached and it was this value that Vrba et al measured.

Careful selection of tracer can eliminate the need for complex (and theoretical) kinetic models when measuring rates of glucose utilization:

Labelling of the glucose molecule.

1. $^{14}$C-labelling of the glucose molecule results in the label occurring in all the glycolytic intermediates, in the intermediates of the citric acid cycle and in carbon dioxide. The $^{14}$C label also appears in newly-formed glucose due to substrate recycling; this results in lower values for glucose utilization than actually occur, as the amount of labelled glucose left in the plasma pool is augmented by the newly formed labelled glucose (30). Thus $^{14}$C-labelled glucose presents problems in measuring glucose utilization.

2. $^3$H-labelling of the glucose molecule is possible for each of the six carbon atoms and the fate of the label depends upon the position of the carbon atom to which it is attached.

(i). ($^3$H-2)-Glucose.

$^3$H on the second carbon atom of the glucose molecule (C2) is lost
There is a hydride shift between C2 of glucose-6-P and C1 of fructose-6-P and, during this process, the hydrogen exchanges extensively with the protons of the medium. In a single transfer, part of the $^3$H from position 2 of glucose-6-P would appear in position 1 of fructose-6-P and part in water. Glucose-6-P and fructose-6-P are reversibly isomerized as shown above. In each isomerization (in either direction) one-half of the hydrogen in position 2 of the aldose and position 1 of ketose will be exchanged with the protons of the medium. After a single isomerization cycle of glucose-6-P to fructose-6-P and back, the $^3$H content at position 2 of glucose will be reduced to 25%. The activity of phosphohexose isomerase is, in general, much higher than the rates of other reactions of the hexose phosphatases and the phosphate esters will be turned over several times before leaving the pool. With high isomerization rates all the $^3$H is lost from C2 of glucose-6-P and C1 of fructose-6-P (30).

Katz and Dunn (30) have shown that, using ($^3$H-2)-glucose, appreciable activity from $^3$H was apparent in water 1-2 minutes after i.v. injection and they found maximal activity in all experiments after 80-100 mins, which remained essentially constant thereafter. The fraction of $^3$H estimated to be in liver glycogen at 100 mins after injection ranged from 0.5-1.0%, while muscle glycogen contained at most 0.5% $^3$H. Thus, Katz and Dunn (30) proposed the use of ($^3$H-2)-glucose to correct for the under-estimate of the rate of glucose utilization caused by recycling of $^{14}$C between glucose and its catabolic products arising in the periphery from the use of (U-14C)-glucose. The basic assumptions underlying this approach were that all catabolism occurs in the periphery, that tritium serves as an irreversible tracer for glucose, and that no detritiation occurs in the liver.
$^3$H on C3 or C5 of glucose is lost during the isomerization between dihydroxyacetone-P and glyceraldehyde-3-P (32, 41). $^3$H on C3 of glucose appears on dihydroxyacetone-P and $^3$H on C5 of glucose appears on glyceraldehyde-3-P.

\[
\begin{align*}
H^+ + OH^- & \quad \xrightarrow{(4) \text{ Triosephosphate isomerase}} \quad H-C-OH \\
& \quad \xrightarrow{(5)} \quad H-C-OH \\
& \quad \xrightarrow{(6)} \quad H-C-OPO_2H_2
\end{align*}
\]

Glyceraldehyde-3-P \quad \xrightarrow{\text{isomerase}} \quad \text{Dihydroxyacetone-P}

In the isomerization of glyceraldehyde-3-P to dihydroxyacetone-P there is a hydride shift from the second carbon atom of glyceraldehyde-3-P to the first carbon atom of dihydroxyacetone-P, and during this process the hydrogen exchanges extensively with protons of the medium. Thus, $^3$H from the C2 of glyceraldehyde (equivalent to $^3$H on C5 of glucose) is lost to water during this isomerization.

Similarly, in the isomerization of dihydroxyacetone-P to glyceraldehyde-3-P, $^3$H from the C1 of dihydroxyacetone-P (equivalent to the $^3$H on C3 of glucose) is lost to water:

\[
\begin{align*}
H^+ + OH^- & \quad \xrightarrow{(3) \text{ Triosephosphate isomerase}} \quad H-C-OH \\
& \quad \xrightarrow{(2)} \quad H-C-OH \\
& \quad \xrightarrow{(1)} \quad H-C-OPO_2H_2
\end{align*}
\]

Dihydroxyacetone-P \quad \xrightarrow{\text{isomerase}} \quad \text{Glyceraldehyde-3-P}

(iii). ($^3$H-4)-Glucose and any $^3$H left on ($^3$H-3)-Glucose.

After the conversion of dihydroxyacetone-P to glyceraldehyde-3-P by glyceraldehyde-3-P dehydrogenase, the C1 of glyceraldehyde-3-P represents both C3 and C4 of glucose. $^3$H in this position of glyceraldehyde-3-P is lost during its oxidation:

\[
\begin{align*}
*H \quad \xrightarrow{(3, 4) \text{ NAD}^+} \quad H-C-OH \\
H-C-OPO_2H_2 \quad \xrightarrow{(2, 5)} \quad \text{Glyceraldehyde-3-P} \\
& \quad \xrightarrow{\text{dehydrogenase}} \quad H-C-OPO_2H_2 \\
& \quad \xrightarrow{\text{NAD}^+ H + H^+} \quad \text{NADH} + *H^+
\end{align*}
\]

Glyceraldehyde-3-P

1,3-Diphosphoglyceric acid
\( ^3 \text{H} \) on C1 and C6 of glucose is retained on C3 of phosphoenolpyruvate and pyruvate as shown below:

\[
\text{COOH} \quad \text{(3,4)} \quad \text{ADP} \quad \text{Pyruvate kinase} \quad \text{COOH} \\
\text{C-OPO}_2 \text{H}_2 \quad \text{(2,5)} \quad \text{Mg}^{++/K^+} \quad \text{H} \\
\text{*H-H} \quad \text{(1,6)} \quad \text{ATP} \\
\]

Phosphoenolpyruvate

Pyruvate

This is, however, rapidly lost by exchange with protons, catalyzed by alanine-2-oxoglutarate transaminase (42), an enzyme with high activity in liver and muscle (32).

The loss of \( ^3 \text{H} \) from C3 of pyruvate occurs during the series of reactions in which pyridoxal phosphate functions as the coenzyme in the transfer of an amino group to the keto acid acceptor (pyruvate) to form alanine:

\[
\text{COOH} \quad \text{(3,4)} \\
\text{C=O} \quad \text{(2,5)} \\
\text{NH}_2-\text{CH} \quad \text{(1,6)} \\
\text{H} \\
\]

Pyruvate

Pyridoxamine-P

\[
\begin{align*}
\text{COOH} & \quad \text{CH}_2\text{PO}_4\text{H}_2 \\
\text{C=O} & \quad \text{CH}_2\text{PO}_4\text{H}_2 \\
\text{H} & \quad \text{CH}_3 \\
\text{H} & \quad \text{CH}_3 \\
\end{align*}
\]

Pyridoxal-P

\[
\begin{align*}
\text{COOH} & \quad \text{OH} \quad \text{H} \quad \text{CH}_2\text{PO}_4\text{H}_2 \\
\text{C=O} & \quad \text{NH}_2 \quad \text{H} \quad \text{CH}_2\text{PO}_4\text{H}_2 \\
\text{H} & \quad \text{H} \\
\text{H} & \quad \text{H} \\
\end{align*}
\]

\( ^3 \text{H}-2 \)-glucose was chosen for this study as the \( ^3 \text{H} \) was irreversibly lost at a very early stage of glucose metabolism (in the isomerization of glucose-6-P and fructose-6-P), which meant that glucose utilization could be measured 'in vivo' without including a measurement of newly formed glucose.

This chapter compares the effects of insulin, tolbutamide, phenformin and Wy 23675 on glucose utilization (as a separate measure from any
rats. This enables a link to be made between the *in vivo* results reported in Chapter 1 and *in vitro* work reported in future chapters, as it permits a determination *in vivo* of whether or not the drug being investigated is affecting tissues involved in glucose utilization.
Materials

Insulin (protamine zinc) was obtained from Macarthys Pharmaceuticals Ltd, Romford, Essex. Alloxan monohydrate was obtained from Koch-Light Laboratories, Colnbrook, Bucks. The sources of other drugs were given earlier (Chapter 1).

Radiolabelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks. Micellar Scintillator NE 260 was obtained from Nuclear Enterprises Ltd, Sighthill, Edinburgh.

Methods

Treatment of Animals.

Rats were male Charles River CD strain (180-220 gms) maintained as described earlier (Chapter 1). Animals were fed ad libitum or fasted for 24 hours prior to the experiment as indicated.

Radiolabelled glucose (10 μCi) was injected into the tail vein of the unanaesthetized rat in 0.2mls of 0.9% saline. Blood samples were taken from the tail at selected times before and after injection and analyzed for blood glucose and plasma insulin levels (see Chapter 1). The administration of drugs is described in the text for each experiment. Alloxan-diabetic animals were treated with alloxan monohydrate (67 mmol/kg intraperitoneally), following a 24 hour fast, 7 days prior to the experiment with radiolabelled glucose when animals were frankly diabetic (as judged by glucosuria).

Treatment of Samples.

For the measurement of radiolabelled glucose in blood, samples were collected from the tail vein into heparinized microfuge tubes and centrifuged in a Beckman Microfuge for 4 minutes at room temperature. 100 μl of the supernatant was freeze-dried in an apparatus consisting of a flask with a single outlet connected to an Edwards High Vacuum Pump ES 50 via two collecting tubes bathed in liquid nitrogen. The residue containing radiolabelled glucose was redissolved in 3 x 1 ml distilled water, mixed with 16 ml of Micellar Scintillator NE 260 (NE 260) and counted for 10 minutes in a Packard Tricarb Liquid Scintillation
The rate of utilization of a substrate can be calculated, following a single injection of radioactive tracer, by determining the decline in specific radioactivity of the substrate in blood with time from the equation:

\[ \text{Rate of Utilization} = \frac{\text{Dose Injected}}{\text{Area under Specific Activity/Time Curve to Infinity}} \]

The conditions necessary for this equation to be valid include the absence of isotopic effects; the replacement of substrate utilized by non-labelled material which passes through the blood; the absence of extensive substrate recycling during the experiment; and the maintenance of the system in a steady state (i.e. the rates into and out of pools and the pool sizes remain constant).

The experiments described here do not conform to steady state conditions as they involve the effects of drugs which lower blood glucose concentrations, causing changes of the rates into and out of pools as well as pool sizes.

Heath and Barton (43) have suggested using the total quantity of label in the plasma glucose pool rather than the specific activity. Using the specific activity (dpm/mmol glucose) to calculate the rates of glucose utilization involves including the value for pool size, and this is only valid when this value remains constant. By using the total quantity of label in the pool the need for a constant pool size is eliminated and so can be applied to non-steady state conditions:

\[ \text{Quantity of Label in Pool} = \text{Specific Radioactivity (dpm/mmol glucose)} \times \text{Glucose Concentration (mmol/ml)} \times \text{Volume of Pool (mls)}. \]

or \[ \text{Quantity of Label} = \text{Radioactivity (dpm)/ml of Plasma} \times \text{Volume of Plasma Pool (mls)}. \]

The quantity of total radioactivity in the plasma pool was calculated on the basis of a glucose volume of distribution equal to the plasma...
volume which, in a 200 gm rat, is 7.97 mls (44). (For practical considerations the quantity of label is normalized to 1.0 at zero time).

It is not possible to calculate a rate of glucose utilization for most of the experiments described in this chapter, since it varies from moment to moment in the non-steady state conditions. Therefore, in each experiment, the quantity of radiolabelled glucose in the plasma pool in drug-treated rats is compared with that in untreated controls.
a). Preliminary Results to Justify the Methodology.

(i). Freeze-Drying Techniques for Separating Radiolabelled Glucose from Plasma.

To check on the losses of labelled glucose during the freeze-drying process, 100 µl samples of rat plasma containing 0.01 µCi \(^{(3}H-2\)-glucose were frozen in 5 ml plastic vials and then freeze-dried. These vials were then washed three times with distilled water (1 ml.) and counted in 16 mls NE 260. Further 100 µl plasma samples containing 0.01 µCi \(^{(3}H-2\)-glucose (the controls) were added directly to 16 mls NE 260 with 3 mls of distilled water in each and counted. The results showed that relatively little loss of counts from \(^{(3}H-2\)-glucose occurred during the freeze-drying process. The average percentage loss on 5 experiments was 8.6% compared with controls.

To check that none of the \(^3H_2O\) (formed by the metabolism of \(^{(3}H-2\)-glucose in the rat) was left in the 5ml plastic vials following freeze-drying, 100 µl samples of rat plasma containing 4 µCi \(^3H_2O\) were freeze-dried and the vials washed with 3 mls. distilled water, which was counted in 16 mls NE 260. Control 100 µl plasma samples containing 4 µCi \(^3H_2O\) were counted in 16 mls. NE 260 with 3 mls. distilled water. Residual counts present after freeze-drying represented only 0.11% (average of 5 experiments) of the total counts recorded from the controls, which were not freeze-dried.

It was concluded that the freeze-drying process was effective in separating \(^{(3}H-2\)-glucose from \(^3H_2O\) in rat plasma.

(ii). \(^{(3}H-2\)-glucose as a Measure of Glucose Utilization.

Plasma levels of \(^{(3}H-2\)-glucose should decrease asymptotically to zero during the course of glucose metabolism in the rat. Figure 2(i) shows a time-course for the decline in plasma \(^{(3}H-2\)-glucose after a single injection at zero time. Radioactivity declined virtually to zero by 150 minutes, which suggests that tritium from \(^{(3}H-2\)-glucose was not recycled into newly-formed glucose. If this were the case as with
appears that the use of \((^3\text{H}-2)\)-glucose as a valid measure of irreversible glucose disposal in the body is justified.

b). The Effects of Agents on Glucose Utilization in Normal, Fed Rats.

The experiments described in this section illustrate the effects of insulin, tolbutamide, phenformin and Wy 23675 on plasma insulin and blood glucose concentrations and on glucose utilization in normal, fed rats.

Glucose utilization is represented by the quantity of radiolabelled glucose remaining in the plasma pool at selected time points. (Thus, the smaller the numerical value, the greater is the glucose utilization). For reasons already discussed, no quantitative value for glucose utilization can be calculated for most of these experiments, so that comparisons have been made between control and test groups and significant changes in glucose utilization are discussed in qualitative terms only.

Insulin was shown to lower blood glucose levels and to enhance glucose utilization as compared with the control group (Table 2(i)). Tolbutamide administered orally elevated plasma insulin levels and subsequently lowered blood glucose concentrations and stimulated glucose utilization (tables 2(ii) and 2(ix)). Phenformin had no effect on plasma insulin levels, blood glucose levels or glucose utilization (Table 2(iii)). This contrasts with the effects of Wy 23675 which, like tolbutamide, elevated plasma insulin levels, lowered glucose levels and stimulated glucose utilization (Table 2(iv)). However, unlike tolbutamide there was no evidence that Wy 23675 markedly elevated plasma insulin levels prior to its effect on glucose utilization. When Wy 23675 was administered by the intravenous route, it no longer elevated plasma insulin levels, but did still lower blood glucose concentrations and stimulate glucose utilization (Table 2(v)). Tolbutamide, administered by the intravenous route, still markedly elevated plasma insulin levels and subsequently lowered blood glucose concentrations and increased glucose utilization (Table 2(vi)).
c). The Effects of Tolbutamide and Wy 23675 on Glucose Utilization in Diazoxide-Pretreated Rats.

In order to further investigate the role that stimulation of insulin release plays in the effects of tolbutamide and Wy 23675 on blood glucose concentrations and glucose utilization, some experiments were carried out using diazoxide to block insulin release.

Oral administration of diazoxide was shown to cause a marked decrease in plasma insulin levels followed by an elevation of blood glucose concentrations and a decrease in glucose utilization. These latter effects can probably be attributed to the lowered plasma insulin levels. The effects of tolbutamide and Wy 23675 were observed in diazoxide-pretreated rats. Wy 23675 lowered blood glucose concentrations and stimulated glucose utilization despite having no effect on plasma insulin levels (Table 2(viii)), while tolbutamide, in a dose that had no effect on plasma insulin levels in the diazoxide-pretreated rat but which was hypoglycaemic in the normal rat, had no effect on blood glucose levels or glucose utilization (Table 2(x)).

d). The Effects of Agents on Glucose Utilisation in Alloxan-Diabetic, Fasted Rats.

The experiments described in this section illustrate the effects of insulin, tolbutamide, phenformin and Wy 23675 on blood glucose levels and glucose utilization in alloxan-diabetic, fasted rats. A comparison of normal, fasted with alloxan-diabetic, fasted rats revealed an apparently reduced rate of glucose utilization in the alloxan-diabetic rats (Table 2(xi)). However, as the normal and alloxan-diabetic rats had constant glucose pool sizes during this comparison, calculations were made to determine the rates of glucose utilization. The method of calculation used followed that of Heath and Barton (43) to calculate the area under a plot of the quantity of $^{3}$H-2-glucose in plasma (normalized to 1.0 at zero time) against time. The mean rate coefficient of glucose utilization was then calculated from the equation:-

\[
\text{Mean Rate Coefficient of Glucose Utilization} = \frac{\text{Dose Injected (1.0)}}{\text{Area under Quantity of } ^{3}\text{H-2-glucose in Plasma v. Time Curve}}
\]
Thus, the mean rate coefficient of glucose utilization for the normal rats was calculated to be 0.362 min$^{-1}$ and for the alloxan-diabetic rats it was 0.303 min$^{-1}$. The mean blood glucose concentrations during the experimental period were 3.58 mmol/l. for the normal rats and 15.51 mmol/l. for the alloxan-diabetic rats. Taking Metcoff and Favour's figure of 7.97 mls. for the plasma volume of a 200 gm. rat (44), this gives a total of 0.029 mmoles of glucose in the plasma pool of the normal rats and 0.124 mmoles of glucose in the plasma pool of the alloxan-diabetic rats. When multiplied by the appropriate rate constants these figures give a mean rate of glucose utilization of 0.011 mmol/min for the normal rats and 0.038 mmol/min for the alloxan-diabetic rats; an increase over the normal rats by 3.5-times.

Insulin was shown to lower blood glucose concentrations and to enhance glucose utilization in fasted, alloxan-diabetic rats (Table 2(xii)). Tolbutamide, in contrast to its effects in the normal rat where it was able to elevate plasma insulin levels, had no significant effects on either blood glucose concentrations or glucose utilization in fasted, alloxan-diabetic rats (Table 2(xiii)). Phenformin lowered blood glucose concentrations and elevated glucose utilization (Table 2(xiv)). Wy 23675 was similarly able to lower blood glucose concentrations and stimulate glucose utilization (Table 2(xv)). The effects of both phenformin and Wy 23675 were apparent in the absence of any measurable effect on plasma insulin levels in alloxan-diabetic rats.

e). The Effects of Futile Cycling in the Measurement of Glucose Utilization.

(3H-2)-glucose was used to measure glucose utilization in these experiments in the belief that the following basic assumptions, made by Katz and Dunn (30), were correct:-

(i). All catabolism occurs in the periphery.

(ii). 3H serves as an irreversible tracer for glucose.

(iii). No detritiation occurs in the liver.
At the end of 1970, Katz et al. published a further paper reconsidering the position of the $^3$H label on glucose for the in vivo estimation of glucose utilization in rats in the light of the fairly recently accepted knowledge concerning substrate recycling (33-37).

Substrate recycling or 'futile cycling' is the cyclic transformation of metabolites in a pathway in which the net balance is the hydrolysis of ATP to ADP and Pi (36). The purpose of these futile cycles is, as yet, unclear; indeed, until recently, their very existence was a subject of some controversy. However, it appears that some of these cycles provide the cell with a valuable regulatory mechanism. This is particularly apparent when recycling occurs at the level of a metabolite, such as glucose-6-P, which participates in the control of metabolic flow.

The purpose of the glucose/glucose-6-P recycling appears to be to allow major changes in glucose uptake or output controlled by the concentration of substrate only, instead of involving a complex control mechanism (36). Newsholme and Start (40) have pointed out that a small change in glucose concentration of 4-6 mM is sufficient to produce a complete change of direction of glucose metabolism in the liver.

Van Hoof et al. (38) have pointed out that there is likely to be recycling in the liver between glucose and glucose-6-P during net hepatic glucose synthesis and that this would invalidate the use of ($^3$H-2)-glucose for the determination of glucose turnover, as tritium would be released without any actual utilization of glucose. Hue and Hers (39) have shown a more rapid decline in the $^3$H/$^{14}$C ratio in glucose after injection of ($^2$H-2, $^1$H-14C)-glucose than after ($^3$H-3, $^1$H-14C)-glucose or ($^3$H-6, $^1$H-14C)-glucose. These ratios would be the same if no tritium was lost in futile cycling.

Thus, the use of ($^3$H-2)-glucose to measure glucose utilization will avoid the problem that arises with the use of ($^1$H-14C)-glucose (namely, that the $^{14}$C will be resynthesized into newly formed glucose and thus give a false picture of glucose utilization), but will give an artificially high measurement of glucose utilization due to the release of tritium during futile cycling of glucose and glucose-6-P.
(\(^3\)H-2)-glucose was derived from hepatic recycling and 85-90% from catabolism in extrahepatic tissues. They showed that the use of (\(^3\)H-2)-glucose would cause an over-estimate of the rate of replacement by about 20%, but would over-estimate the recycling of \(^{14}\)C by a factor of two as compared with (\(^3\)H-3)- or (\(^3\)H-6)-glucose. On the other hand, futile cycles accounted for only a very small fraction of detritiation from (\(^3\)H-3)- or (\(^3\)H-5)-glucose. For this reason, they have suggested the use of glucose labelled with tritium in one of these positions, rather than position 2, to measure glucose utilization in the rat in vivo.

Because the use of (\(^3\)H-2)-glucose gives false measurements, many of the experiments, reported in this section, will have an over-estimate of the rate of glucose utilization. However, it is not the basic rate that is of prime interest in these experiments so much as the changes that occur with the drugs investigated. Each such test result is run against a control, so that the change brought about by the drug can be measured with respect to it. For this reason, the over-estimate of the rate of glucose utilization caused by the futile cycling between glucose and glucose-6-P and the loss of tritium from (\(^3\)H-2)-glucose is not important provided the drugs do not directly affect the futile cycling. It only becomes important to the results of this investigation if the hormones or drugs being investigated act to increase or decrease futile cycling.

If, however, they act on one of the enzymes involved in the futile cycle to cause an increase or decrease in net flow of substrate through the cycle, then any \(^3\)H lost from the labelled substrate, above or below the level of the controls, would reflect an increase or decrease in substrate flow.

As several workers (32,39) now recommend the use of (\(^3\)H-3)-glucose to measure glucose utilization 'in vivo' rather than (\(^3\)H-2)-glucose, several experiments have been repeated using (\(^3\)H-3)-glucose and the results compared with those obtained from experiments with (\(^3\)H-2)-glucose.
A direct comparison of the rates of glucose utilization as measured by \( ^3\text{H}-2\)-glucose and \( ^3\text{H}-3\)-glucose was made in normal rats (Table 2(xvi)). As these rats were in a 'steady state', calculations were made to determine values for glucose utilization (these were carried out as described earlier in the comparison of normal and alloxan-diabetic rats). The mean rate of glucose utilization in the rats using \( ^3\text{H}-2\)-glucose was 0.013 mmol/min and in the rats using \( ^3\text{H}-3\)-glucose was 0.011 mmol/min. Thus, the use of \( ^3\text{H}-2\)-glucose over-estimated the rate of glucose utilization by approximately 18% - this is in line with the findings of Katz et al (32). However, further work with \( ^3\text{H}-3\)-glucose has shown that insulin and Wy 23675 have similar qualitative effects on glucose utilization in both normal and alloxan-diabetic rats whether this was measured with \( ^3\text{H}-2\)-glucose (Tables 2(i), 2(iv), 2(xii), 2(xv)) or with \( ^3\text{H}-3\)-glucose (Tables 2(xvii), 2(xviii), 2(xix), 2(xx)). Thus, the fact that \( ^3\text{H}-2\)-glucose over-estimates the rate of glucose utilization does not alter the validity of the findings concerning the qualitative effects of the drugs on glucose utilization.
THE USE OF RADIOLABELLED GLUCOSE TO MEASURE THE EFFECTS OF SELECTED AGENTS ON GLUCOSE UTILIZATION IN THE NORMAL AND ALLOXAN-DIABETIC RAT

'IN VIVO'.

**Figure No.**  
**Title**

2(i)  'In Vivo' Loss of \(^3\text{H}-2\)-glucose from the Blood of the Normal Rat with Time.

2(ii) Effect of Insulin on the Quantity of \(^3\text{H}-2\)-glucose Remaining in the Plasma Pool.

**Table No.**  
**Title**

2(i) Effect of Intramuscular Insulin on Glucose and \(^3\text{H}-2\)-glucose Levels in Blood of the Normal Rat.

2(ii) Effect of Oral Tolbutamide on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Normal Rat.

2(iii) Effect of Oral Phenformin on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Normal Rat.

2(iv) Effect of Oral Wy 23675 on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Normal Rat.

2(v) Effect of Intravenous Wy 23675 on Glucose, Insulin and \(^3\text{H}-2\)-Glucose Levels in the Blood of the Normal Rat.

2(vi) Effect of Intravenous Tolbutamide on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Normal Rat.

2(vii) Effect of Oral Diazoxide on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Normal Rat.

2(viii) Effect of Oral Wy 23675 on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Diazoxide-Treated Rat.

2(ix) Effect of Oral Tolbutamide on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Normal Rat.

2(x) Effect of Oral Tolbutamide on Glucose, Insulin and \(^3\text{H}-2\)-glucose Levels in the Blood of the Diazoxide-Treated Rat.
<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(xi)</td>
<td>Effect of Alloxan Pretreatment on Glucose and ((^3\text{H}-2))-glucose Levels in the Blood of the Normal Rat.</td>
</tr>
<tr>
<td>2(xii)</td>
<td>Effect of Intramuscular Insulin on Glucose and ((^3\text{H}-2))-glucose Levels in the Blood of the Alloxan-Diabetic Rat.</td>
</tr>
<tr>
<td>2(xiii)</td>
<td>Effect of Oral Tolbutamide on Glucose and ((^3\text{H}-2))-glucose Levels in the Blood of the Alloxan-Diabetic Rat.</td>
</tr>
<tr>
<td>2(xiv)</td>
<td>Effect of Oral Phenformin on Glucose and ((^3\text{H}-2))-glucose Levels in the Blood of the Alloxan-Diabetic Rat.</td>
</tr>
<tr>
<td>2(xv)</td>
<td>Effect of Oral Wy 23675 on Glucose and ((^3\text{H}-2))-glucose Levels in the Blood of the Alloxan-Diabetic Rat.</td>
</tr>
<tr>
<td>2(xvi)</td>
<td>Comparison of the Use of ((^3\text{H}-2))-glucose and ((^3\text{H}-3))-glucose to Measure Glucose Utilization in Normal, Untreated Rats <em>in Vivo</em>.</td>
</tr>
<tr>
<td>2(xvii)</td>
<td>Effect of Intramuscular Insulin on Glucose and ((^3\text{H}-3))-glucose Levels in the Blood of the Normal Rat.</td>
</tr>
<tr>
<td>2(xviii)</td>
<td>Effect of Oral Wy 23675 on Glucose, Insulin and ((^3\text{H}-3))-glucose Levels in the Blood of the Normal Rat.</td>
</tr>
<tr>
<td>2(xix)</td>
<td>Effect of Intramuscular Insulin on Glucose and ((^3\text{H}-3))-glucose Levels in the Blood of the Alloxan-Diabetic Rat.</td>
</tr>
<tr>
<td>2(xx)</td>
<td>Effect of Oral Wy 23675 on Glucose and ((^3\text{H}-3))-glucose Levels in the Blood of the Alloxan-Diabetic Rat.</td>
</tr>
</tbody>
</table>
IN VIVO LOSS OF $^{3}H_{-2}$-GLUCOSE FROM BLOOD OF NORMAL RAT WITH TIME

Six male rats (190-210 g) were injected intravenously with 10 µCi $^{3}H_{-2}$-glucose in 0.2 ml volume. 0.3 ml blood samples were taken from the tail at 30, 60, 90, 120, 150 and 180 mins after injection and centrifuged in a Beckman Microfuge. 100 µls of plasma from each sample was freeze-dried in 5 ml plastic vials. These were then washed three times with distilled water (1 ml) and counted in 16 mls NE 260.

![Graph showing the loss of $^{3}H_{-2}$-glucose with time](image)

- **$^{3}H_{-2}$-glucose**
- (dpm/100 µls freeze-dried plasma)

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dpm/100 µls freeze-dried plasma)</td>
<td>7000</td>
<td>5000</td>
<td>2000</td>
<td>1000</td>
<td>500</td>
<td>250</td>
<td>125</td>
</tr>
</tbody>
</table>
Rats were injected with 10 μCi (²H-2)-glucose i.v. and either 0.1 ml of protamine zinc insulin (4 units/rat i.m.) or 0.1 ml saline i.m. (controls) at zero time. 0.3 ml blood samples were taken at the time intervals shown and used for glucose analysis and for measuring (²H-2)-glucose. Values represent the mean of 6 animals with the standard error shown in brackets.

The quantity of total radioactivity in the pool was calculated as described in Methods and normalized to that theoretically present at the time of injection (10 μCi=22×10⁶dpm). Statistical differences between control and insulin-treated groups were examined by Student's t-Test:

* p<0.05  *** p<0.001

<table>
<thead>
<tr>
<th>Time after (²H-2)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>(²H-2)-Glucose Levels (dpm/100 μls freeze-dried plasma)</th>
<th>Quantity of (²H-2)-Glucose in Plasma Pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>4.29±0.11</td>
<td>4.95±0.28</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>4.51±0.22</td>
<td>5.01±0.22</td>
<td>79041±6321</td>
</tr>
<tr>
<td>3</td>
<td>4.24±0.06</td>
<td>4.90±0.11</td>
<td>84304±7119</td>
</tr>
<tr>
<td>5</td>
<td>4.18±0.17</td>
<td>5.12±0.22</td>
<td>72167±7001</td>
</tr>
<tr>
<td>7</td>
<td>4.46±0.17</td>
<td>4.84±0.17</td>
<td>62469±5936</td>
</tr>
<tr>
<td>9</td>
<td>4.40±0.11</td>
<td>4.68±0.22</td>
<td>65395±6333</td>
</tr>
<tr>
<td>30</td>
<td>4.57±0.17</td>
<td>4.40±0.22</td>
<td>8430±621</td>
</tr>
<tr>
<td>60</td>
<td>4.29±0.22</td>
<td>3.74±0.11</td>
<td>3062±219</td>
</tr>
<tr>
<td>90</td>
<td>4.80±0.17</td>
<td>2.64±0.11</td>
<td>1023±289</td>
</tr>
<tr>
<td>Time after ((3\text{H}-2\text{-}))-Glucose (mins)</td>
<td>Quantity Normalized to (1\cdot0) at Zero Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1\cdot000</td>
<td>1\cdot000</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0\cdot286 (±0\cdot023)</td>
<td>0\cdot312 (±0\cdot030)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0\cdot305 (±0\cdot026)</td>
<td>0\cdot302 (±0\cdot035)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0\cdot261 (±0\cdot025)</td>
<td>0\cdot271 (±0\cdot030)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0\cdot226 (±0\cdot022)</td>
<td>0\cdot240 (±0\cdot008)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0\cdot237 (±0\cdot030)</td>
<td>0\cdot186 (±0\cdot017)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>0\cdot031 (±0\cdot003)</td>
<td>0\cdot018*** (±0\cdot002)</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0\cdot011 (±0\cdot002)</td>
<td>0\cdot003*** (±0\cdot001)</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0\cdot004 (±0\cdot001)</td>
<td>0\cdot001*** (±0\cdot001)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2(ii)

**EFFECT OF ORAL TOLBUTAMIDE ON GLUCOSE, INSULIN AND \(^{3}H\)-2-GLUCOSE LEVELS IN BLOOD OF THE NORMAL RAT.**

Rats were injected with 10 µCi \(^{3}H\)-2-glucose i.v. and were dosed orally with either tolbutamide (0.2 mmol/kg) or HPMC (1 ml/100 gm body weight) at zero time. Blood samples were taken at the time intervals shown and used for plasma insulin and glucose analysis and for measuring \(^{3}H\)-2-glucose. The quantity of radioactivity in the glucose pool was calculated as described in Methods and normalized to 1.0 at the time of injection. The results are expressed as the mean ± the standard error for 6 animals. Statistical significance was determined by the Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
(NM = not measured ; Tolb. = Tolbutamide group)

<table>
<thead>
<tr>
<th>Time after (^{3}H)-2-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (µUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (^{3}H)-2-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>4.73 (±0.28)</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>NM</td>
<td>4.90 (±0.17)</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>NM</td>
<td>5.12 (±0.22)</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>NM</td>
<td>5.01 (±0.22)</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>NM</td>
<td>4.84 (±0.28)</td>
</tr>
<tr>
<td>9</td>
<td>5 (±4)</td>
<td>90**</td>
<td>4.84 (±0.06)</td>
</tr>
<tr>
<td>30</td>
<td>-2 (±7)</td>
<td>114***</td>
<td>4.62 (±0.11)</td>
</tr>
<tr>
<td>60</td>
<td>10 (±8)</td>
<td>60*</td>
<td>4.95 (±0.28)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>NM</td>
<td>4.79 (±0.22)</td>
</tr>
</tbody>
</table>

At zero time, the plasma insulin levels were 41 (±6) and 35 (±5)µU/ml for the control and tolbutamide groups, respectively.
### Table 2(iii)

**EFFECT OF ORAL PHENFORMIN ON GLUCOSE, INSULIN AND ($^{3}\text{H-2}$)-GLUCOSE LEVELS IN BLOOD OF THE NORMAL RAT.**

Rats were injected with 10 μCi ($^{3}\text{H-2}$)-glucose i.v. and were orally dosed with either phenformin (0.2 mmol/kg) or with HPMC (1 ml/100 gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean ± the standard error for 6 animals. Statistical significance was determined by Student's t-Test - no significant differences were found between the control and phenformin groups in this experiment. (NM = not measured).

<table>
<thead>
<tr>
<th>Time after ($^{3}\text{H-2}$)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of ($^{3}\text{H-2}$)-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Phenformin</td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>4.35 (±0.28)</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>NM</td>
<td>4.51 (±0.17)</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>NM</td>
<td>4.57 (±0.22)</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>NM</td>
<td>4.29 (±0.17)</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>NM</td>
<td>4.73 (±0.33)</td>
</tr>
<tr>
<td>9</td>
<td>20 (17)</td>
<td>12 (18)</td>
<td>4.46 (±0.39)</td>
</tr>
<tr>
<td>30</td>
<td>13 (18)</td>
<td>2 (14)</td>
<td>4.35 (±0.17)</td>
</tr>
<tr>
<td>60</td>
<td>2 (11)</td>
<td>4 (14)</td>
<td>4.62 (±0.22)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>NM</td>
<td>4.68 (±0.33)</td>
</tr>
</tbody>
</table>

At zero time, the plasma insulin levels were 37 (±3) and 31 (±7) μU/ml for the control and phenformin groups, respectively.
Table 2(iv)

**EFFECT OF ORAL WY 23675 ON GLUCOSE, INSULIN AND (3H-2)-GLUCOSE LEVELS IN THE BLOOD OF THE NORMAL RAT.**

Rats were injected with 10 μCi (3H-2)-glucose i.v, and were orally dosed with either WY 23675 (0.2 mmol/kg) or with HPMC (1 ml/100 gm body weight) at zero time. Other details are as in Table 2(ii), The results are expressed as the mean for 6 animals with the standard error in brackets. Statistical significance was determined by Student's t-Test:

** p<0.01 ; *** p<0.001 (NM : not measured)

<table>
<thead>
<tr>
<th>Time after (3H-2)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (3H-2)-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>5.12 ±0.33</td>
<td>4.79 ±0.22</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>4.95 ±0.22</td>
<td>4.90 ±0.22</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>5.17 ±0.39</td>
<td>5.06 ±0.28</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>4.84 ±0.28</td>
<td>4.79 ±0.17</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>5.06 ±0.17</td>
<td>4.90 ±0.39</td>
</tr>
<tr>
<td>9</td>
<td>5 (±17)</td>
<td>4.90 ±0.22</td>
<td>4.73 ±0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.73 ±0.22</td>
<td>4.02 ±0.33</td>
</tr>
<tr>
<td>30</td>
<td>2 (±11)</td>
<td>4.73 ±0.22</td>
<td>4.02 ±0.33</td>
</tr>
<tr>
<td>60</td>
<td>13 (±12)</td>
<td>4.90 ±0.17</td>
<td>3.08*** ±0.28</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>5.06 ±0.28</td>
<td>2.86*** ±0.39</td>
</tr>
</tbody>
</table>

At zero time the plasma insulin levels were 32 (±4) and 35 (±6) μU/ml for the control and WY 23675 groups, respectively.
Table 2(v)

<table>
<thead>
<tr>
<th>Time after (3H-2)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/1)</th>
<th>Quantity of (3H-2)-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>4.79 (±0.28)</td>
<td>5.06 (±0.17)</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>4.90 (±0.39)</td>
<td>4.84 (±0.22)</td>
</tr>
<tr>
<td></td>
<td>NM</td>
<td>5.06 (±0.17)</td>
<td>4.95 (±0.17)</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>4.84 (±0.22)</td>
<td>4.79 (±0.33)</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>4.90 (±0.39)</td>
<td>4.68 (±0.22)</td>
</tr>
<tr>
<td>7</td>
<td>7 (±2)</td>
<td>4.57 (±0.33)</td>
<td>3.96 (±0.28)</td>
</tr>
<tr>
<td></td>
<td>15 (±15)</td>
<td>4.84 (±0.22)</td>
<td>5.25** (±0.33)</td>
</tr>
<tr>
<td>30</td>
<td>12 (±21)</td>
<td>4.90 (±0.39)</td>
<td>2.42*** (±0.28)</td>
</tr>
<tr>
<td>60</td>
<td>11 (±11)</td>
<td>4.73 (±0.28)</td>
<td>2.04*** (±0.44)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>4.73 (±0.28)</td>
<td>2.04*** (±0.44)</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 41 (±2) and 37 (±3) μU/ml for the control and Wy 23675 groups, respectively.
Table 2(vi)

EFFECT OF INTRAVENOUS TOLBUTAMIDE ON GLUCOSE, INSULIN AND (3H-2)-GLUCOSE LEVELS IN THE BLOOD OF THE NORMAL RAT.

Rats were injected with 10 μCi (3H-2)-glucose i.v. and were injected i.v. with either tolbutamide 0.08 mmol/kg br with 0.9% saline (0.1 ml/100 gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: * p<0.05 ; ** p<0.01 ; *** p<0.001 (NM : not measured)

<table>
<thead>
<tr>
<th>Time after Change in Plasma Blood Glucose Quantity of (3H-2)-Glucose relative to Glucose normalized to Glucose Zero Time (mmol/1) at Zero Time (μUnits/ml)</th>
<th>Control</th>
<th>Tolb.</th>
<th>Control</th>
<th>Tolb.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>4.90 (±0.17)</td>
<td>4.73 (±0.22)</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>NM</td>
<td>5.01 (±0.33)</td>
<td>4.20 (±0.33)</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>NM</td>
<td>4.79 (±0.28)</td>
<td>4.79 (±0.39)</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>NM</td>
<td>4.90 (±0.22)</td>
<td>4.84 (±0.28)</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>NM</td>
<td>4.68 (±0.22)</td>
<td>4.68 (±0.28)</td>
</tr>
<tr>
<td>9</td>
<td>10 (±10)</td>
<td>105* (±31)</td>
<td>4.95 (±0.22)</td>
<td>4.29 (±0.33)</td>
</tr>
<tr>
<td>30</td>
<td>17 (±18)</td>
<td>85** (±18)</td>
<td>4.73 (±0.33)</td>
<td>3.47 (±0.39)</td>
</tr>
<tr>
<td>60</td>
<td>4 (±14)</td>
<td>62** (±14)</td>
<td>4.68 (±0.22)</td>
<td>2.81*** (±0.33)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>NM</td>
<td>5.01 (±0.28)</td>
<td>2.48*** (±0.33)</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 32 (±7) and 27 (±5) μUnits/ml for the control and tolbutamide groups, respectively.
Table 2(vii)

**EFFECT OF ORAL DIAZOXIDE ON GLUCOSE, INSULIN AND (3H-2)-GLUCOSE LEVELS IN THE BLOOD OF THE NORMAL RAT.**

Rats were injected with 10 uCi (3H-2)-glucose i.v. and were orally dosed with either diazoxide (1.6 mmol/kg) or with HPMC (1ml/100 gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001  (NM : not measured)

<table>
<thead>
<tr>
<th>Time after (3H-2)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (µUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (3H-2)-Glucose normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Diazoxide</td>
<td>Control Diazoxide</td>
<td>Control Diazoxide</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>4.62 (±0.17)</td>
<td>1.000</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>4.73 (±0.22)</td>
<td>0.323</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>4.57 (±0.28)</td>
<td>0.319</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>4.35 (±0.22)</td>
<td>0.298</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>4.57 (±0.22)</td>
<td>0.298</td>
</tr>
<tr>
<td>9 (±5)</td>
<td>-12*</td>
<td>4.68 (±0.17)</td>
<td>0.254</td>
</tr>
<tr>
<td>30</td>
<td>-28**</td>
<td>4.29 (±0.28)</td>
<td>0.037</td>
</tr>
<tr>
<td>60</td>
<td>-31***</td>
<td>4.35 (±0.38)</td>
<td>0.021</td>
</tr>
<tr>
<td>90</td>
<td>-</td>
<td>4.57 (±0.22)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 39 (±4) and 40 (±6) µUnits/ml for the control and diazoxide groups, respectively.
Table 2(viii)

EFFECT OF ORAL WY 23675 ON GLUCOSE, INSULIN AND ($^3$H-2)-GLUCOSE LEVELS IN THE BLOOD OF THE DIAZOXIDE-TREATED RAT.

All rats used were dosed with diazoxide (1.6 mmol/kg p.o.) 1 hour prior to the intravenous administration of ($^3$H-2)-glucose (10 μCi) and oral dosing with either Wy 23675 (0.2 mmol/kg) or with HPMC (1 ml/100 gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: *p<0.05; **p<0.01; ***p<0.001 (NM: not measured)

<table>
<thead>
<tr>
<th>Time after ($^3$H-2)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of ($^3$H-2)-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>7.59 (±0.60)</td>
<td>8.03 (±0.50)</td>
</tr>
<tr>
<td>1</td>
<td>NM (±7)</td>
<td>7.81 (±0.54)</td>
<td>8.36 (±0.61)</td>
</tr>
<tr>
<td>3</td>
<td>NM (±7)</td>
<td>7.54 (±0.55)</td>
<td>8.03 (±0.44)</td>
</tr>
<tr>
<td>5</td>
<td>NM (±7)</td>
<td>8.03 (±0.72)</td>
<td>8.14 (±0.39)</td>
</tr>
<tr>
<td>7</td>
<td>NM (±7)</td>
<td>8.31 (±0.61)</td>
<td>7.65 (±0.55)</td>
</tr>
<tr>
<td>9</td>
<td>1 (±7)</td>
<td>8.14 (±0.39)</td>
<td>7.59 (±0.39)</td>
</tr>
<tr>
<td>30</td>
<td>4 (±5)</td>
<td>8.47 (±0.72)</td>
<td>6.66* (±0.28)</td>
</tr>
<tr>
<td>60</td>
<td>1 (±8)</td>
<td>8.69 (±0.50)</td>
<td>4.90*** (±0.33)</td>
</tr>
<tr>
<td>90</td>
<td>NM (±7)</td>
<td>8.56 (±0.60)</td>
<td>5.12** (±0.72)</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 6 (±4) and 9 (±7) μUnits/ml for the control and Wy 23675 group, respectively.
### Table 2(ix)

**EFFECT OF ORAL TOLBUTAMIDE ON GLUCOSE, INSULIN AND (**H-2 **) GLUCOSE LEVELS IN THE BLOOD OF THE NORMAL RAT.**

Rats were injected with 10 μCi (**H-2 **) glucose i.v. and were orally dosed with either tolbutamide (0.05mmol/kg) or with HPMC (1 ml/100 gm. body weight) at zero time. Other details are as in Table 2(ii). Results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test:  
* p<0.05; ** p<0.01; *** p<0.001 (NM : not measured)

(Tolb. = Tolbutamide)

<table>
<thead>
<tr>
<th>Time after (<strong>H-2</strong>) Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (<strong>H-2</strong>) Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>5.50</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>NM</td>
<td>5.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.39)</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>NM</td>
<td>5.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.56)</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>NM</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.22)</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>NM</td>
<td>5.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.33)</td>
</tr>
<tr>
<td>9</td>
<td>61**</td>
<td></td>
<td>5.50</td>
</tr>
<tr>
<td></td>
<td>(±21)</td>
<td></td>
<td>(±0.22)</td>
</tr>
<tr>
<td>30</td>
<td>70**</td>
<td></td>
<td>5.34</td>
</tr>
<tr>
<td></td>
<td>(±29)</td>
<td></td>
<td>(±0.33)</td>
</tr>
<tr>
<td>60</td>
<td>41</td>
<td></td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>(±31)</td>
<td></td>
<td>(±0.39)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>NM</td>
<td>5.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.33)</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 40 (±2) and 36 (±4) μUnits/ml for the control group and tolbutamide group, respectively.
Table 2(x)

**EFFECT OF ORAL TOLBUTAMIDE ON GLUCOSE, INSULIN AND \(^{3}H-2\)-GLUCOSE LEVELS IN THE BLOOD OF THE DIAZOXIDE-TREATED RAT.**

All rats used were dosed with diazoxide (1.6mmol/kg p.o.) 1 hr prior to the intravenous administration of \(^{3}H-2\)-glucose (10 μCi) and oral dosing with either tolbutamide (0.05mmol/kg) or with HPMC (1 ml/100gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean ± the standard error for 6 animals. Statistical significance was determined by Student's t-Test - no significant differences were found between the control and tolbutamide groups. (NM : not measured) (Tolb. = Tolbutamide)

<table>
<thead>
<tr>
<th>Time after (^{3}H-2)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (^{3}H-2)-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>7.98</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>NM</td>
<td>8.47</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>NM</td>
<td>7.81</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>NM</td>
<td>8.75</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>NM</td>
<td>8.97</td>
</tr>
<tr>
<td>9</td>
<td>-6</td>
<td>2</td>
<td>8.80</td>
</tr>
<tr>
<td></td>
<td>(±7)</td>
<td>(±7)</td>
<td>(±0.66)</td>
</tr>
<tr>
<td>30</td>
<td>-7</td>
<td>1</td>
<td>8.20</td>
</tr>
<tr>
<td></td>
<td>(±5)</td>
<td>(±11)</td>
<td>(±0.66)</td>
</tr>
<tr>
<td>60</td>
<td>2</td>
<td>5</td>
<td>8.64</td>
</tr>
<tr>
<td></td>
<td>(±5)</td>
<td>(±5)</td>
<td>(±0.44)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>NM</td>
<td>8.42</td>
</tr>
<tr>
<td></td>
<td>(±0.39)</td>
<td>(±0.61)</td>
<td>(±0.003)</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 7 (±4) and 4 (±3) μUnits/ml for the control and tolbutamide groups, respectively.
Table 2(xi)

EFFECT OF ALLOXAN PRETREATMENT ON GLUCOSE AND (2H-2)-GLUCOSE LEVELS IN
THE BLOOD OF THE NORMAL RAT.

Alloxan-diabetic rats were treated as described in Methods and were used 8 days afterwards. Both control and alloxan-diabetic rats were fasted for 24 hrs prior to the experiment and were then injected with 10 μCi (2H-2)-glucose i.v. (zero time). Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: ** p<0.01 ; *** p<0.001.

<table>
<thead>
<tr>
<th>Time after (2H-2)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (2H-2)-glucose in Plasma normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Alloxan</td>
</tr>
<tr>
<td>0</td>
<td>3.58±0.28</td>
<td>15.51***</td>
</tr>
<tr>
<td>1</td>
<td>3.91±0.39</td>
<td>16.23***</td>
</tr>
<tr>
<td>3</td>
<td>4.02±0.28</td>
<td>15.07***</td>
</tr>
<tr>
<td>5</td>
<td>3.74±0.50</td>
<td>15.84***</td>
</tr>
<tr>
<td>7</td>
<td>3.63±0.33</td>
<td>16.34***</td>
</tr>
<tr>
<td>9</td>
<td>3.80±0.22</td>
<td>16.61***</td>
</tr>
<tr>
<td>30</td>
<td>3.47±0.44</td>
<td>16.01***</td>
</tr>
<tr>
<td>60</td>
<td>3.63±0.33</td>
<td>15.90***</td>
</tr>
<tr>
<td>90</td>
<td>3.25±0.39</td>
<td>16.34***</td>
</tr>
</tbody>
</table>

At zero time, plasma insulin levels were 39 (±5) μUnits/ml for the controls but were below the sensitivity of the radio-immunoassay in the alloxan-treated rats.
Table 2(xii)

EFFECT OF INTRAMUSCULAR INSULIN ON GLUCOSE AND \((^{3}H-2)\)-GLUCOSE LEVELS IN THE BLOOD OF THE ALLOXAN-DIABETIC RAT.

Rats, alloxan-diabetic for 8 days (see Methods), were fasted for 24 hrs prior to the experiment and were then injected with 10 μCi \((^{3}H-2)\)-glucose i.v. and with either protamine zinc insulin (4 units/rat i.m. or with 0.9% saline (0.1 ml/100 gm. body weight i.m) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: * p<0.05 ; ** p<0.01 ; *** p<0.001 (NM : not measured)

<table>
<thead>
<tr>
<th>Time after ((^{3}H-2))-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of ((^{3}H-2))-glucose in Plasma normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
</tr>
<tr>
<td>0</td>
<td>17.93</td>
<td>18.92</td>
</tr>
<tr>
<td>1</td>
<td>18.65</td>
<td>17.66</td>
</tr>
<tr>
<td>3</td>
<td>18.76</td>
<td>18.48</td>
</tr>
<tr>
<td>5</td>
<td>18.40</td>
<td>19.20</td>
</tr>
<tr>
<td>7</td>
<td>16.94</td>
<td>17.05</td>
</tr>
<tr>
<td>9</td>
<td>17.16</td>
<td>15.79</td>
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<tr>
<td>30</td>
<td>17.93</td>
<td>11.72*</td>
</tr>
<tr>
<td>60</td>
<td>18.32</td>
<td>7.92***</td>
</tr>
<tr>
<td>90</td>
<td>17.55</td>
<td>9.19**</td>
</tr>
</tbody>
</table>
Table 2(xiii)

EFFECT OF ORAL TOLBUTAMIDE ON GLUCOSE AND (3H-2)-GLUCOSE LEVELS IN THE
BLOOD OF THE ALLOXAN DIABETIC RAT.

Rats, alloxan-diabetic for 8 days (see Methods), were fasted for 24 hrs prior to the experiment and were then injected with 10 μCi (3H-2)-glucose i.v. and were orally dosed with either tolbutamide (2 mmol/kg) or with HPMC (1 ml/100 gm. body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for the 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test - no significant differences were found between the control and tolbutamide groups.

<table>
<thead>
<tr>
<th>Time after (3H-2)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/1)</th>
<th>Quantity of (3H-2)-glucose in Plasma normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Tolbutamide</td>
</tr>
<tr>
<td>0</td>
<td>20.63</td>
<td>21.56</td>
</tr>
<tr>
<td></td>
<td>(+2.09)</td>
<td>(-1.71)</td>
</tr>
<tr>
<td>1</td>
<td>19.64</td>
<td>19.91</td>
</tr>
<tr>
<td></td>
<td>(+2.20)</td>
<td>(-1.65)</td>
</tr>
<tr>
<td>3</td>
<td>19.91</td>
<td>20.79</td>
</tr>
<tr>
<td></td>
<td>(+1.76)</td>
<td>(-1.49)</td>
</tr>
<tr>
<td>5</td>
<td>20.19</td>
<td>20.96</td>
</tr>
<tr>
<td></td>
<td>(+1.21)</td>
<td>(-1.54)</td>
</tr>
<tr>
<td>7</td>
<td>21.56</td>
<td>20.46</td>
</tr>
<tr>
<td></td>
<td>(+1.65)</td>
<td>(-1.76)</td>
</tr>
<tr>
<td>9</td>
<td>21.34</td>
<td>22.66</td>
</tr>
<tr>
<td></td>
<td>(+2.26)</td>
<td>(-2.09)</td>
</tr>
<tr>
<td>30</td>
<td>19.47</td>
<td>21.40</td>
</tr>
<tr>
<td></td>
<td>(+1.05)</td>
<td>(+2.26)</td>
</tr>
<tr>
<td>60</td>
<td>20.35</td>
<td>21.67</td>
</tr>
<tr>
<td></td>
<td>(+1.49)</td>
<td>(+2.15)</td>
</tr>
<tr>
<td>90</td>
<td>21.18</td>
<td>22.33</td>
</tr>
<tr>
<td></td>
<td>(+1.71)</td>
<td>(+1.43)</td>
</tr>
</tbody>
</table>

Plasma insulin levels were below the sensitivity of the radio-immunoassay in both groups.
**Table 2(xiv)**

**EFFECT OF ORAL PHENFORMIN ON GLUCOSE AND (3H-2)-GLUCOSE LEVELS IN THE BLOOD OF THE ALLOXAN-DIABETIC RAT.**

Rats, alloxan-diabetic for 8 days (see Methods), were fasted for 24 hrs prior to the experiment and were then injected with 10 μCi (3H-2)-glucose i.v. and were orally dosed with either phenformin (0.2 mmol/kg) or with HPMC (1 ml/100 gm. body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: * p<0.05 ; ** p<0.01 ; *** p<0.001 (NM: not measured)

<table>
<thead>
<tr>
<th>Time after (3H-2)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (3H-2)-glucose in Plasma normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Phenformin</td>
</tr>
<tr>
<td>0</td>
<td>21.78±1.71</td>
<td>17.93±1.49</td>
</tr>
<tr>
<td>1</td>
<td>15.57±1.49</td>
<td>17.44±2.15</td>
</tr>
<tr>
<td>3</td>
<td>17.16±1.76</td>
<td>18.59±1.32</td>
</tr>
<tr>
<td>5</td>
<td>16.89±1.05</td>
<td>18.81±1.65</td>
</tr>
<tr>
<td>7</td>
<td>16.06±1.38</td>
<td>17.55±1.21</td>
</tr>
<tr>
<td>9</td>
<td>17.38±1.32</td>
<td>15.68±1.60</td>
</tr>
<tr>
<td>30</td>
<td>17.99±1.93</td>
<td>13.42±1.16</td>
</tr>
<tr>
<td>60</td>
<td>16.45±1.21</td>
<td>11.22±1.75</td>
</tr>
<tr>
<td>90</td>
<td>16.67±0.99</td>
<td>11.88±1.54</td>
</tr>
</tbody>
</table>

Plasma insulin levels were below the sensitivity of the radio-immunoassay in both groups.
**Table 2(xv)**

**EFFECT OF ORAL WY 23675 ON GLUCOSE AND (3H-2)-GLUCOSE LEVELS IN THE BLOOD OF THE ALLOXAN-DIABETIC RAT.**

Rats, alloxan-diabetic for 8 days (see Methods), were fasted for 24 hrs prior to the experiment and were then injected with 10 μCi (3H-2)-glucose i.v., and were orally dosed with either Wy 23675(0.2mmol/kg) or with HPMC (1 ml/100 gm. body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: * p<0.05 ; *** p<0.001 (NM : not measured)

<table>
<thead>
<tr>
<th>Time after (3H-2)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (3H-2)-glucose in Plasma normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Wy 23675 Control Wy 23675</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15.51±1.21 16.72±1.65</td>
<td>1.000 1.000</td>
</tr>
<tr>
<td>1</td>
<td>16.17±1.71 16.01±1.10</td>
<td>0.318±0.057 0.338±0.059</td>
</tr>
<tr>
<td>3</td>
<td>15.79±1.60 15.90±1.49</td>
<td>0.311±0.030 0.322±0.042</td>
</tr>
<tr>
<td>5</td>
<td>17.27±1.49 17.11±1.38</td>
<td>0.301±0.031 0.291±0.034</td>
</tr>
<tr>
<td>7</td>
<td>16.34±1.38 16.50±1.55</td>
<td>0.280±0.030 0.250±0.031</td>
</tr>
<tr>
<td>9</td>
<td>16.83±1.32 16.06±1.76</td>
<td>0.257±0.026 0.218±0.025</td>
</tr>
<tr>
<td>30</td>
<td>16.50±1.27 12.05±1.49</td>
<td>0.063±0.007 0.032±0.003</td>
</tr>
<tr>
<td>60</td>
<td>15.51±1.49 9.35±1.38</td>
<td>0.050±0.005 0.020±0.002</td>
</tr>
<tr>
<td>90</td>
<td>16.17±1.71 10.12±1.21</td>
<td>0.043±0.005 0.007±0.001</td>
</tr>
</tbody>
</table>

Plasma insulin levels were below the sensitivity of the radio-immunoassay in both groups.
Rats were injected with either 10 μCi (3H-2)-glucose or 10 μCi (3H-3)-glucose/200 gm body weight i.v. Blood samples were taken at the time intervals shown and used for plasma insulin and glucose analysis and for measuring 3H-labelled glucose. Other details are as in Table 2(ii). The results are expressed as the mean for 6 animals with the standard error of the mean in brackets. Statistical significance was determined by Student's t-Test: * p<0.05 ; ** p<0.01 (Gl = Glucose ; NM = not measured)

<table>
<thead>
<tr>
<th>Time after 3H-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (μUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of 3H-Gl.* Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(3H-2)-Gl*</td>
<td>(3H-3)-Gl*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3H-2)-Gl*</td>
<td>(3H-3)-Gl*</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>4.35 (±0.28)</td>
<td>1.000</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>4.57 (±0.33)</td>
<td>0.923 (±0.39)</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>4.91 (±0.44)</td>
<td>0.932 (±0.030)</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>4.73 (±0.39)</td>
<td>0.929 (±0.031)</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>4.29 (±0.28)</td>
<td>0.926 (±0.039)</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>4.62 (±0.17)</td>
<td>0.935 (±0.021)</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>4.72 (±0.33)</td>
<td>0.936 (±0.025)</td>
</tr>
<tr>
<td>60</td>
<td>7</td>
<td>4.95 (±0.22)</td>
<td>0.918 (±0.002)</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>4.57 (±0.39)</td>
<td>0.900 (±0.01)</td>
</tr>
</tbody>
</table>

At zero time, the plasma insulin levels were 38 (±7) and 40 (±6) μU/ml for the (3H-2)-glucose and (3H-3)-glucose injected groups, respectively.
Rats were injected with 10 μCi \(^3\)H-3-glucose i.v. and with either 0.1 ml of protamine zinc insulin (4 units/rat i.m.) or 0.1 ml saline i.m. (controls) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean ± the standard error for 6 animals. Statistical significance was determined by Student's t-Test:

** p(0.01 ; *** p(0.001 (NM = not measured)

<table>
<thead>
<tr>
<th>Time after ((^3)H-3)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of ((^3)H-3)-glucose in Plasma Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
</tr>
<tr>
<td>0</td>
<td>4.57±0.28</td>
<td>5.01±0.33</td>
</tr>
<tr>
<td>1</td>
<td>4.46±0.44</td>
<td>4.73±0.28</td>
</tr>
<tr>
<td>3</td>
<td>4.73±0.28</td>
<td>4.62±0.33</td>
</tr>
<tr>
<td>5</td>
<td>4.62±0.22</td>
<td>5.01±0.17</td>
</tr>
<tr>
<td>7</td>
<td>4.95±0.33</td>
<td>4.84±0.17</td>
</tr>
<tr>
<td>9</td>
<td>4.84±0.28</td>
<td>4.73±0.22</td>
</tr>
<tr>
<td>30</td>
<td>4.57±0.17</td>
<td>3.96±0.22</td>
</tr>
<tr>
<td>60</td>
<td>4.73±0.28</td>
<td>3.30***</td>
</tr>
<tr>
<td>90</td>
<td>4.79±0.22</td>
<td>2.31***</td>
</tr>
</tbody>
</table>
Table 2(xviii)

EFFECT OF ORAL WY 23675 ON GLUCOSE, INSULIN AND (3H-3)-GLUCOSE LEVELS IN BLOOD OF THE NORMAL RAT.

Rats were injected with 10 µCi (3H-3)-glucose i.v. and were dosed orally with either WY 23675 (0.2 mmol/kg) or with HPMC (1 ml/100 gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean ± the standard error for 6 animals. Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001 (NM = not measured)

<table>
<thead>
<tr>
<th>Time after (3H-3)-Glucose (mins)</th>
<th>Change in Plasma Insulin relative to Zero Time (µUnits/ml)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (3H-3)-Glucose Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
<td>Control Wy 23675</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
</tr>
<tr>
<td>1</td>
<td>NM</td>
<td>NM</td>
<td>0.308</td>
</tr>
<tr>
<td>3</td>
<td>NM</td>
<td>NM</td>
<td>0.305</td>
</tr>
<tr>
<td>5</td>
<td>NM</td>
<td>NM</td>
<td>0.289</td>
</tr>
<tr>
<td>7</td>
<td>NM</td>
<td>NM</td>
<td>0.269</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
<td>14</td>
<td>0.252</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>41*</td>
<td>0.045</td>
</tr>
<tr>
<td>60</td>
<td>6</td>
<td>63**</td>
<td>0.031</td>
</tr>
<tr>
<td>90</td>
<td>NM</td>
<td>NM</td>
<td>0.017</td>
</tr>
</tbody>
</table>

At zero time, the plasma insulin levels were 32 (±5) and 38 (±3) µU/ml for the control and WY 23675 groups, respectively.
Table 2 (xix)

**EFFECT OF INTRAMUSCULAR INSULIN ON GLUCOSE AND \( ^{3}H-3 \)GLUCOSE LEVELS IN THE BLOOD OF THE ALLOXAN-DIABETIC RAT.**

Rats, alloxan-diabetic for 8 days (see Methods), were fasted for 24 hrs prior to the experiment and were then injected with 10 μCi \( ^{3}H-3 \)-glucose i.v. and with either protamine zinc insulin (4 μunits/rat i.m.) or with 0.9% saline (0.1 ml/100 gm body weight) at zero time. Other details are as in Table 2 (ii). The results are expressed as the mean ± the standard-error for 6 animals. Statistical significance was determined by Student's t-Test:

** p<0.01 ; *** p<0.001 (NM = not measured)

<table>
<thead>
<tr>
<th>Time after ( ^{3}H-3 )-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of ( ^{3}H-3 )-glucose in Plasma Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Insulin</td>
</tr>
<tr>
<td>0</td>
<td>19.36 (± 2.09)</td>
<td>18.48 (± 2.42)</td>
</tr>
<tr>
<td>1</td>
<td>20.49 (± 2.31)</td>
<td>19.31 (± 2.04)</td>
</tr>
<tr>
<td>3</td>
<td>19.20 (± 1.82)</td>
<td>18.59 (± 1.43)</td>
</tr>
<tr>
<td>5</td>
<td>19.69 (± 2.59)</td>
<td>19.03 (± 1.76)</td>
</tr>
<tr>
<td>7</td>
<td>20.46 (± 1.65)</td>
<td>17.71 (± 1.98)</td>
</tr>
<tr>
<td>9</td>
<td>19.09 (± 2.04)</td>
<td>16.89 (± 1.71)</td>
</tr>
<tr>
<td>30</td>
<td>19.86 (± 1.98)</td>
<td>10.23** (± 1.71)</td>
</tr>
<tr>
<td>60</td>
<td>19.69 (± 1.87)</td>
<td>7.98*** (± 1.54)</td>
</tr>
<tr>
<td>90</td>
<td>19.09 (± 1.60)</td>
<td>7.32*** (± 1.76)</td>
</tr>
</tbody>
</table>
Table 2

EFFECT OF ORAL WY 23675 ON GLUCOSE AND (³H-3)-GLUCOSE LEVELS IN THE BLOOD OF THE ALLOXAN-DIABETIC RAT.

Rats, alloxan-diabetic for 8 days (see Methods), were fasted for 24 hours prior to the experiment and were then injected with 10 μCi (³H-3)-glucose i.v, and were dosed orally with either WY 23675 (0.2 mmol/kg) or with HPMC (1 ml/100 gm body weight) at zero time. Other details are as in Table 2(ii). The results are expressed as the mean ± the standard error for 6 animals. Statistical significance was determined by Student's t-Test:
* p<0.05 ; ** p<0.01 ; *** p<0.001 (NM = not measured)

<table>
<thead>
<tr>
<th>Time after (³H-3)-Glucose (mins)</th>
<th>Blood Glucose Levels (mmol/l)</th>
<th>Quantity of (³H-3)-glucose in Plasma Normalized to 1.0 at Zero Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Wy 23675</td>
</tr>
<tr>
<td></td>
<td>(±2.31)</td>
<td>(±1.49)</td>
</tr>
<tr>
<td>0</td>
<td>17.66</td>
<td>17.05</td>
</tr>
<tr>
<td></td>
<td>(±2.04)</td>
<td>(±1.82)</td>
</tr>
<tr>
<td>1</td>
<td>16.83</td>
<td>17.39</td>
</tr>
<tr>
<td></td>
<td>(±2.48)</td>
<td>(±1.65)</td>
</tr>
<tr>
<td>3</td>
<td>18.37</td>
<td>18.48</td>
</tr>
<tr>
<td></td>
<td>(±2.48)</td>
<td>(±1.65)</td>
</tr>
<tr>
<td>5</td>
<td>19.09</td>
<td>17.88</td>
</tr>
<tr>
<td></td>
<td>(±1.93)</td>
<td>(±1.38)</td>
</tr>
<tr>
<td>7</td>
<td>18.48</td>
<td>17.16</td>
</tr>
<tr>
<td></td>
<td>(±1.82)</td>
<td>(±1.98)</td>
</tr>
<tr>
<td>9</td>
<td>17.82</td>
<td>16.67</td>
</tr>
<tr>
<td></td>
<td>(±2.09)</td>
<td>(±1.76)</td>
</tr>
<tr>
<td>30</td>
<td>17.49</td>
<td>12.43*</td>
</tr>
<tr>
<td></td>
<td>(±1.97)</td>
<td>(±1.16)</td>
</tr>
<tr>
<td>60</td>
<td>18.48</td>
<td>10.51*</td>
</tr>
<tr>
<td></td>
<td>(±2.09)</td>
<td>(±1.34)</td>
</tr>
<tr>
<td>90</td>
<td>18.04</td>
<td>9.63**</td>
</tr>
<tr>
<td></td>
<td>(±1.76)</td>
<td>(±1.65)</td>
</tr>
</tbody>
</table>

Plasma insulin levels were below the sensitivity of the radio-immunoassay in both groups.
The results described in this chapter have shown some effects of the hypoglycaemic agents—insulin, tolbutamide, phenformin and Wy 23675—on glucose utilization in vivo in normal, diazoxide-pretreated and alloxan-diabetic rats. The use of the three different models (ie: normal, diazoxide-pretreated and alloxan-diabetic rats) has enabled differences in the modes of action of tolbutamide, phenformin and Wy 23675 to be highlighted.

Diazoxide-pretreatment resulted in an inhibition of insulin release from the pancreatic β-cells and therefore a fall in plasma insulin levels, which, in turn, resulted in a hyperglycaemia and an increase in the plasma glucose pool size. The injection of the same quantity of radiolabelled glucose into normal and diazoxide-treated rats resulted in a considerably greater dilution of radioactivity in the larger glucose pool of the diazoxide-treated rats than in the normal rats and, therefore, the quantity of radiolabelled glucose lost from the pool was very much less in the diazoxide-treated rats than in the normal rats, even though the rates of glucose utilization could have been the same in both situations. This emphasizes the necessity for the careful selection of controls when using comparative rates of radiolabelled glucose disposal to measure glucose utilization. For this reason, the effects of the hypoglycaemic agents were studied in diazoxide-pretreated rats, rather than investigating the effects of diazoxide-treatment in rats treated with the hypoglycaemic agents.

Physiologically, diazoxide-pretreatment represented a situation of hyperglycaemia, in which insulin release was blocked.

Alloxan-treatment, on the other hand, resulted in a marked hyperglycaemia through the destruction of the pancreatic β-cells, which caused a fall in plasma insulin to levels below the limits of detection by radioimmunoassay. Alloxan-diabetes caused an apparent decrease in the quantity of radiolabelled glucose lost from the plasma pool compared with normal controls. However, when the mean rate coefficient for glucose utilization was multiplied by the mean quantity of glucose in the pool
diabetic rats, was approximately 3.5-fold the value for the normal rats. Thus, alloxan-diabetes resulted in an increase in the rate of glucose utilization as compared with the fasted, control rats. Baker et al (7) have similarly reported increase in the rate of glucose utilization in fasted, alloxan-diabetic rats, which was accompanied by an increase in the rate of glucose oxidation to carbon dioxide as compared with normal, fasted rats. The high rate of glucose utilization in the fasted, alloxan-diabetic rat may be a reflection of the high circulating concentrations of glucose, in contrast to normal rats, where fasting results in a considerable reduction in blood glucose concentrations.

The rates of glucose utilization in control animals in the present experiments were similar to those reported by Heath and Corney (45) - 0.004 mmol glucose/min/100 gm rat body weight; cf. 0.005 mmol/min/100 gm rat body weight in the present work.

Insulin, in these experiments, increased the rate of glucose utilization in both normal and alloxan-diabetic rats. Tolbutamide, by increasing plasma insulin levels in normal rats, stimulated glucose utilization indirectly. Thus, when insulin release was inhibited by diazoxide or in the alloxan-diabetic rat, tolbutamide had no effect on glucose utilization.

The results for tolbutamide contrast with those of phenformin, which had no effects on either insulin release or glucose utilization in the normal rat, while in the alloxan-diabetic rat it caused an increase in the rates of glucose utilization. These findings for phenformin in the rat are in contrast to those of Searle and Cavalieri (46) in human subjects. From studies using $^{14}$C-glucose, these authors concluded that glucose turnover is increased by phenformin, and that this effect is greater in non-diabetic than in diabetic subjects. They reason that the increased removal of glucose in the non-diabetic is compensated by increased gluconeogenesis, and hypoglycaemia does not occur. In the diabetic, gluconeogenesis is already maximal and cannot be increased, resulting in a fall in blood glucose levels. However, the studies of Altschuld and
gluconeogenesis from lactate and glycerol is inhibited by phenformin. Other workers have made similar findings in both the guinea-pig (48) and rat livers (49,50). The results reported in this chapter indicate, that at least part of the hypoglycaemia caused by phenformin in the alloxan-diabetic rat is due to an increase in glucose utilization.

The results for the novel hypoglycaemic agent, Wy 23675, contrast with both those from the sulphonylurea, tolbutamide, and from the biguanide, phenformin. Unlike phenformin, Wy 23675 increased glucose utilization in the normal, fed rat. However, oral administration of Wy 23675 also increased plasma insulin levels and so, in this way resembled tolbutamide in its action. When Wy 23675 was administered intravenously it had no effect on plasma insulin levels, but still increased glucose utilization. Thus, the action of Wy 23675 differed from both phenformin and tolbutamide in the normal rat. Further confirmation of the insulin-independent action of Wy 23675 on glucose utilization was obtained in experiments in which diazoxide blocked insulin release. Under these conditions, Wy 23675 still stimulated glucose utilization. Wy 23675 also stimulated glucose utilization in the alloxan-diabetic rat, as did phenformin. Thus, a comparison of the effects of Wy 23675 on glucose utilization with those of phenformin and tolbutamide, reveals novel aspects of Wy 23675 action.

In all cases, the stimulatory effects of these agents on glucose utilization paralleled their effectiveness in causing hypoglycaemia. It can be concluded, therefore, that this stimulation is part of the mechanism by which these agents induce a fall in blood glucose levels.
References.


Chapter 3.
Chapter 3

THE EFFECTS OF INSULIN, TOLBUTAMIDE, PHENFORMIN AND Wy 23675 ON GLUCOSE UTILIZATION BY RAT HEMIDIAPHRAGMS

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This section contains the results of experiments carried out with rat hemidiaphragms (prepared from normal and alloxan-diabetic rats) to investigate the effects of selected hypoglycaemic agents upon glucose utilization, lactate and pyruvate production, and glycogen synthesis.

Results of incubations carried out with normal rat hemidiaphragms showed the following effects of these agents:

1. Insulin stimulated glucose uptake and glycogen synthesis in a dose-related manner, but had no effect on lactate and pyruvate production.

2. Tolbutamide had no effect on glucose uptake, lactate and pyruvate production, or glycogen synthesis.

3. Phenformin had no effect on any of the parameters measured at therapeutic concentrations (2μM), but did stimulate glucose uptake and lactate production and lower glycogen levels at higher concentrations (200 μM).

4. Wy 23675 did stimulate glucose uptake and glycogen production at concentrations that would be reached with hypoglycaemic doses of the drug, but had no effect on lactate and pyruvate production.

Results from incubations carried out with hemidiaphragms from alloxan-diabetic rats showed the following effects of these agents:

1. Hemidiaphragms from alloxan-diabetic rats had lower rates of glucose uptake and glycogen synthesis than those from normal rats, although the production of lactate and pyruvate was not significantly altered.

2. Insulin significantly stimulated glucose uptake and glycogen synthesis but had no effect on lactate and pyruvate production. The effect of insulin was to bring the levels of glucose utilization and glycogen synthesis to those of hemidiaphragms prepared from normal rats.

3. Tolbutamide, as with normal rat hemidiaphragms, had no effect on glucose uptake, lactate and pyruvate production, or glycogen synthesis.

4. Phenformin stimulated glucose utilization and lactate production and lowered glycogen levels, but had no effect on pyruvate production. There was a significant phenformin effect at ten-fold lower concentrations with hemidiaphragms from alloxan-diabetic rats than from normal rats.
5. Wy 23675, like insulin, elevated glucose uptake and glycogen synthesis by hemidiaphragms from alloxan-diabetic rats to levels normally seen with untreated rat hemidiaphragms. Wy 23675 had no effect on lactate or pyruvate production.

The results shown in this chapter support the in vivo findings on glucose utilization described in Chapter 2. Differences in the actions of Wy 23675 and the other drugs studied are further illustrated; and their possible mechanisms of action are discussed.
Agents that lower blood glucose levels can do so by either stimulating glucose uptake and/or inhibiting glucose synthesis. Muscle is a major site of glucose utilization and probably the two most used animal models to study this are the rat hemidiaphragm and the isolated perfused rat hindquarter preparation.

Insulin has been shown to accelerate both the utilization of glucose \((1-10,27)\) and the synthesis of glycogen \((1,7,8)\) by the isolated rat hemidiaphragm, but has no effect on lactate production \((5,8)\). Studies in the isolated perfused rat hindquarter have shown similar effects of insulin \((16-24)\).

The studies with radiolabelled glucose in Chapter 2, have shown that tolbutamide has no effect on glucose utilization unless insulin is available and that phenformin has no effect on glucose utilization in the normal rat, but does stimulate it in alloxan-diabetic rats.

Feldman and Lebovitz \((25)\) have presented evidence that tolbutamide may potentiate insulin effects on skeletal muscle metabolism. They used intact mouse diaphragm and showed that tolbutamide in vitro did not increase 2-deoxyglucose uptake nor did it augment insulin-mediated 2-deoxyglucose uptake. However, when tolbutamide was administered in vivo up to twelve days before sacrifice a significant potentiation of the insulin effect on 2-deoxyglucose uptake was observed.

Biguanides have been reported to stimulate glucose uptake by hemidiaphragms from normal rats \((3,12-15)\), but the concentrations of drug used in these studies were well above those obtained in the blood following therapeutic doses. Frayn and Adnitt \((7)\) have exposed rat hemidiaphragms to therapeutic concentrations of metformin \((10 \mu g/ml)\) and failed to show any effect of the drug on glucose uptake, either in the absence or presence of insulin, although they showed a stimulation at higher non-therapeutic concentrations. In hemidiaphragms from alloxan-diabetic rats, they showed that metformin enhanced the insulin-stimulation of glucose uptake. Work with isolated perfused hindquarter preparations from normal rats have shown a lack of any effect of biguanides in therapeutic
of insulin (21, 22, 26).

The work described in this chapter compares the effects of insulin, tolbutamide and phenformin with those of Wy 23675 on glucose uptake, lactate and pyruvate production, and glycogen synthesis in both normal and alloxan-diabetic rat hemidiaphragms.
The sources of all drugs used were given in earlier chapters.

Enzymes used for lactate and pyruvate analyses were obtained from Sigma, Kingston-upon-Thames, Surrey.

Methods.

The preparation of isolated rat hemidiaphragms was based upon the method of Vallance-Owen and Hurlock (27). Hemidiaphragms were prepared in the same way from both normal and alloxan-diabetic (blood glucose levels greater than 16.5 mmol/litre after 48 hrs) rats.

110-140 gm male rats were fasted for 18 hrs prior to being killed by decapitation. The abdomen was opened and the diaphragm was carefully removed and placed in ice-cold glucose-free Krebs-bicarbonate buffer equilibrated with 95% O₂/5% CO₂ (pH 7.4). The diaphragm, while so immersed, was divided into two approximately equal halves, the thick posterior portion being discarded. For the incubations, one hemidiaphragm was used as a control and the other as a test. Each hemidiaphragm was gently blotted, weighed, and placed in a small beaker containing 2 ml of the incubation medium.

Incubations were carried out at 37°C for 90 minutes and shaken at 90 oscillations/minute. Each beaker was gassed throughout the incubation period with 95% O₂/5% CO₂. The incubation medium was Krebs-bicarbonate buffer equilibrated with 95% O₂/5% CO₂ (pH 7.4), containing 16.5 mmol/litre glucose.

Drugs were added to the incubation medium prior to the addition of the hemidiaphragms. At the end of the incubations, the rat hemidiaphragms were removed from the medium and aliquots of the medium were thoroughly mixed with an equal volume of ice-cold 10% trichloroacetic acid (TCA) and centrifuged at 2000g for 5 minutes.

Samples were assayed for glucose on the Autoanalyzer and for lactate and pyruvate, using enzymatic techniques (28).
On removal from the medium, the rat hemidiaphragm was immediately homogenized in 10 ml of ice-cold 5% TCA and centrifuged at 2000g for 10 minutes. 1 ml samples of the supernatant were treated as described by Pfleiderer (29) to hydrolyze the glycogen to glucose and the glucose concentration was then determined on the Autoanalyzer.

Insulin caused a dose-related increase in glucose uptake by the rat hemidiaphragm in these experiments - Table 3(i). There was no significant change in lactate or pyruvate production, but insulin did increase glycogen synthesis by the hemidiaphragm. In comparison, tolbutamide had no effect on any of the parameters measured in concentrations as high as 20mM - Table 3(i). Phenformin did cause an increase in glucose uptake by the rat hemidiaphragm, although this was only measurable at the concentration of 200µM - Table 3(i). This increase in glucose uptake was accompanied by an increase in lactate production and a fall in glycogen synthesis. Wy 23675, like insulin, caused a dose-related increase in glucose uptake, which was accompanied by an increase in glycogen synthesis with no apparent change in lactate or pyruvate production - Table 3(i).


Hemidiaphragms from fasted, normal rats (mean blood glucose levels of 3.20±0.17 mmol/l.) were compared with hemidiaphragms from fasted, alloxan-diabetic rats (mean blood glucose levels of 17.21±3.65 mmol/l.). The rate of glucose uptake in the alloxan-diabetic rat hemidiaphragm was reduced by approximately 50% compared with that of the normal rat and glycogen synthesis was reduced by about 70% - Table 3(ii). There was a slight reduction in lactate production in the alloxan-diabetic rat hemidiaphragm, but this was not statistically significant.

Insulin stimulated glucose uptake by approximately 100% in the alloxan-diabetic rat hemidiaphragm - Table 3(iii), increasing it to a similar level to that recorded in the normal rat hemidiaphragm. Similarly, insulin stimulated glycogen synthesis by 180% to bring it back to the levels recorded in normal rat hemidiaphragm. There were no significant
observed in the normal rat hemidiaphragm, had no effect on any of the parameters measured in alloxan-diabetic rat hemidiaphragms - Table 3(iii).

Phenformin, as seen in the normal rat hemidiaphragm, stimulated glucose uptake, decreased glycogen synthesis and stimulated lactate production - Table 3(iii). Wy 23675, also as seen in the normal rat hemidiaphragm, stimulated glucose uptake and glycogen formation, while having no effect on lactate or pyruvate production - Table 3(iii).
Chapter 3

THE EFFECT OF INSULIN, TOLBUTAMIDE, PHENFORMIN AND WY23675 ON GLUCOSE UPTAKE, LACTATE AND PYRUVATE PRODUCTION, AND GLYCOGEN SYNTHESIS BY HEMIDIAPHRAGMS PREPARED FROM NORMAL AND ALLOXAN-DIABETIC RATS.

Table No. Title

3(i) Glucose Uptake, Lactate and Pyruvate Production, and Glycogen Synthesis by Hemidiaphragms Prepared from Normal, Fasted Rats.


3(iii) Glucose Uptake, Lactate and Pyruvate Production, and Glycogen Synthesis by Hemidiaphragms Prepared from Alloxan-Diabetic, Fasted Rats.
GLUCOSE UPTAKE, LACTATE AND PYRUVATE PRODUCTION, AND GLYCOGEN SYNTHESIS
BY HEMIDIAPHRAGMS PREPARED FROM NORMAL, FASTED RATS.

<table>
<thead>
<tr>
<th></th>
<th>Glucose Uptake (μmoles/gm wet weight/hr)</th>
<th>Lactate Production</th>
<th>Pyruvate Production</th>
<th>Glycogen Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin 10 μU/ml</td>
<td>9.81±1.02</td>
<td>10.12±0.98</td>
<td>0.97±0.11</td>
<td>3.15±0.80</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+0.73</td>
<td>-1.83</td>
<td>-0.20</td>
<td>+1.70</td>
</tr>
<tr>
<td>Control</td>
<td>10.54±1.89</td>
<td>8.29±0.91</td>
<td>0.77±0.09</td>
<td>4.85±1.21</td>
</tr>
<tr>
<td>Insulin 100 μU/ml</td>
<td>16.97±2.77</td>
<td>11.34±1.12</td>
<td>0.89±0.10</td>
<td>6.21±1.07</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+6.70*</td>
<td>+2.55</td>
<td>+0.05</td>
<td>+3.29*</td>
</tr>
<tr>
<td>Control</td>
<td>8.89±0.92</td>
<td>11.23±1.21</td>
<td>1.06±0.11</td>
<td>3.31±0.65</td>
</tr>
<tr>
<td>Insulin 1000 μU/ml</td>
<td>20.70±2.54</td>
<td>13.38±1.42</td>
<td>1.12±0.13</td>
<td>6.96±1.28</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+11.81**</td>
<td>+2.15</td>
<td>+0.06</td>
<td>+3.65**</td>
</tr>
<tr>
<td>2. Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tolbutamide 0.2mM</td>
<td>7.43±0.69</td>
<td>6.01±0.74</td>
<td>0.72±0.08</td>
<td>2.99±0.27</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+0.58</td>
<td>-0.14</td>
<td>-0.08</td>
<td>+0.12</td>
</tr>
<tr>
<td>Control</td>
<td>8.01±0.92</td>
<td>5.87±0.66</td>
<td>0.84±0.08</td>
<td>3.11±0.43</td>
</tr>
<tr>
<td>Tolbutamide 2mM</td>
<td>10.66±0.97</td>
<td>7.26±0.77</td>
<td>0.74±0.09</td>
<td>3.82±0.44</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+0.71</td>
<td>+0.13</td>
<td>-0.03</td>
<td>+0.55</td>
</tr>
<tr>
<td>Control</td>
<td>12.32±1.76</td>
<td>9.74±1.09</td>
<td>0.92±0.10</td>
<td>4.22±0.46</td>
</tr>
<tr>
<td>Tolbutamide 20mM</td>
<td>11.17±1.29</td>
<td>8.66±1.12</td>
<td>0.89±0.09</td>
<td>3.97±0.41</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>-1.15</td>
<td>-1.08</td>
<td>-0.03</td>
<td>-0.25</td>
</tr>
<tr>
<td>3. Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenformin 2μM</td>
<td>15.33±2.47</td>
<td>10.12±1.21</td>
<td>0.99±0.09</td>
<td>5.16±0.63</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>-1.55</td>
<td>-0.75</td>
<td>-0.03</td>
<td>-0.06</td>
</tr>
<tr>
<td>Control</td>
<td>13.78±1.52</td>
<td>9.37±0.89</td>
<td>0.96±0.09</td>
<td>5.22±0.54</td>
</tr>
<tr>
<td>Phenformin 20μM</td>
<td>12.03±1.34</td>
<td>7.84±0.82</td>
<td>1.06±0.09</td>
<td>4.05±0.42</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+0.28</td>
<td>-1.45</td>
<td>+0.12</td>
<td>+0.59</td>
</tr>
<tr>
<td>Control</td>
<td>8.36±0.94</td>
<td>6.66±0.59</td>
<td>0.84±0.07</td>
<td>3.24±0.34</td>
</tr>
<tr>
<td>Phenformin 200μM</td>
<td>11.27±1.01</td>
<td>9.25±0.87</td>
<td>1.06±0.09</td>
<td>1.92±0.26</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+2.91**</td>
<td>+2.59*</td>
<td>+0.22</td>
<td>-1.32*</td>
</tr>
</tbody>
</table>

Glycogen levels are expressed in terms of μmoles glucose equivalents/gm wet weight/hr.

Each figure is the mean ± the standard error of 6 observations.

Statistically significant changes from the appropriate controls were determined by Student's t-Test:—

* p<0.05 ; ** p<0.01 ; *** p<0.001
<table>
<thead>
<tr>
<th></th>
<th>Glucose Uptake</th>
<th>Lactate Production</th>
<th>Pyruvate Production</th>
<th>Glycogen Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>4. Control</td>
<td>9.42±1.01</td>
<td>10.12±1.14</td>
<td>1.26±0.13</td>
<td>4.46±0.52</td>
</tr>
<tr>
<td>Wy 23675 20μM</td>
<td>10.67±0.98</td>
<td>9.22±0.89</td>
<td>0.94±0.09</td>
<td>3.46±0.39</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+1.25</td>
<td>-0.90</td>
<td>-0.32</td>
<td>-0.79</td>
</tr>
<tr>
<td>Control</td>
<td>10.33±0.99</td>
<td>9.77±0.98</td>
<td>1.11±0.09</td>
<td>3.87±0.41</td>
</tr>
<tr>
<td>Wy 23675 200μM</td>
<td>14.42±1.14</td>
<td>10.01±0.99</td>
<td>0.94±0.09</td>
<td>5.02±0.50</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+4.09*</td>
<td>+0.24</td>
<td>-0.17</td>
<td>+1.15</td>
</tr>
<tr>
<td>Control</td>
<td>8.76±0.86</td>
<td>9.14±1.02</td>
<td>0.89±0.07</td>
<td>4.13±0.39</td>
</tr>
<tr>
<td>Wy 23675 2mM</td>
<td>17.26±1.34</td>
<td>8.96±0.86</td>
<td>0.92±0.10</td>
<td>7.33±0.81</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+8.5***</td>
<td>-0.18</td>
<td>+0.03</td>
<td>+3.20**</td>
</tr>
</tbody>
</table>

Glycogen levels are expressed in terms of μmoles glucose equivalents/gm wet weight/hr.

Each figure is the mean ± the standard error of 6 observations.

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
COMPARISON OF GLUCOSE UPTAKE, LACTATE AND PYRUVATE PRODUCTION, AND GLYCOGEN SYNTHESIS BY HEMIDIAPHRAGMS PREPARED FROM FASTED, NORMAL AND ALLOXAN-DIABETIC RATS.

As it was not possible in this comparison to use one hemidiaphragm from a rat for a control and the other for a test incubation, twelve hemidiaphragms were prepared from six untreated rats and compared with twelve hemidiaphragms prepared from six alloxan-diabetic rats. Rats were made alloxan-diabetic by treatment with alloxan monohydrate (0.7 mmol/kg i.p.) two days prior to the 18-hr fast. Rats with blood glucose levels in excess of 16.5 mmol/l. were used.

<table>
<thead>
<tr>
<th>Glucose Uptake</th>
<th>Lactate Production</th>
<th>Pyruvate Production</th>
<th>Glycogen Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µmoles/gm wet weight/hr)</td>
<td>(µmoles/gm wet weight/hr)</td>
<td>(µmoles/gm wet weight/hr)</td>
<td>(µmoles glucose equivalents/gm wet weight/hr)</td>
</tr>
<tr>
<td>Normal</td>
<td>10.01±1.02</td>
<td>11.76±1.14</td>
<td>0.96±0.10</td>
</tr>
<tr>
<td>Alloxan-Diabetic</td>
<td>4.84±0.52</td>
<td>8.97±0.92</td>
<td>0.84±0.07</td>
</tr>
<tr>
<td>Difference</td>
<td>-5.17**</td>
<td>-2.79</td>
<td>-0.12</td>
</tr>
</tbody>
</table>

Glycogen levels are expressed in terms of µmoles glucose equivalents/gm wet weight/hr.

Each figure is the mean ± the standard error of 12 observations.

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
GLUCOSE UPTAKE, LACTATE AND PYRUVATE PRODUCTION, AND GLYCOGEN SYNTHESIS BY HEMIDIDAPHRAGMS PREPARED FROM ALLOXAN-DIABETIC, FASTED RATS.

<table>
<thead>
<tr>
<th></th>
<th>Glucose Uptake</th>
<th>Lactate Production</th>
<th>Pyruvate Production</th>
<th>Glycogen Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µmoles/gm wet weight/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin 10µU/ml</td>
<td>5.87±0.64</td>
<td>8.74±0.96</td>
<td>1.06±0.09</td>
<td>1.46±0.22</td>
</tr>
<tr>
<td>Difference relative to</td>
<td>+1.88</td>
<td>+0.68</td>
<td>-0.12</td>
<td>+0.61</td>
</tr>
<tr>
<td>Control</td>
<td>7.75±0.61</td>
<td>9.42±1.12</td>
<td>0.94±0.11</td>
<td>2.07±0.26</td>
</tr>
<tr>
<td><strong>2. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tolbutamide 0.2mM</td>
<td>3.76±0.42</td>
<td>7.47±0.86</td>
<td>0.84±0.10</td>
<td>1.23±0.32</td>
</tr>
<tr>
<td>Difference relative to</td>
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<td>+1.74</td>
<td>+0.12</td>
<td>+0.53</td>
</tr>
<tr>
<td>Control</td>
<td>4.25±0.51</td>
<td>9.21±1.21</td>
<td>0.96±0.09</td>
<td>1.76±0.27</td>
</tr>
<tr>
<td><strong>3. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenformin 2µM</td>
<td>4.18±0.44</td>
<td>7.79±0.82</td>
<td>0.91±0.08</td>
<td>1.62±0.29</td>
</tr>
<tr>
<td>Difference relative to</td>
<td>+0.89</td>
<td>-0.45</td>
<td>-0.04</td>
<td>+0.57</td>
</tr>
<tr>
<td>Control</td>
<td>5.07±0.65</td>
<td>8.34±0.92</td>
<td>0.87±0.07</td>
<td>2.19±0.34</td>
</tr>
<tr>
<td><strong>2. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tolbutamide 2mM</td>
<td>5.17±0.52</td>
<td>8.65±0.92</td>
<td>0.97±0.11</td>
<td>2.72±0.54</td>
</tr>
<tr>
<td>Difference relative to</td>
<td>-0.29</td>
<td>-0.74</td>
<td>+0.04</td>
<td>-0.56</td>
</tr>
<tr>
<td>Control</td>
<td>4.88±0.56</td>
<td>7.89±0.88</td>
<td>1.01±0.09</td>
<td>2.16±0.36</td>
</tr>
<tr>
<td><strong>3. Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenformin 20µM</td>
<td>5.77±0.68</td>
<td>8.44±0.96</td>
<td>0.94±0.09</td>
<td>2.47±0.36</td>
</tr>
<tr>
<td>Difference relative to</td>
<td>+3.08*</td>
<td>+1.92</td>
<td>+0.27</td>
<td>-0.91*</td>
</tr>
<tr>
<td>Control</td>
<td>8.85±0.87</td>
<td>10.35±1.14</td>
<td>1.21±0.13</td>
<td>1.56±0.14</td>
</tr>
<tr>
<td><strong>3. Control</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Phenformin 200µM</td>
<td>3.91±0.52</td>
<td>6.92±0.68</td>
<td>0.77±0.08</td>
<td>2.07±0.37</td>
</tr>
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<td>Difference relative to</td>
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<td>+2.82*</td>
<td>+0.21</td>
<td>-1.11*</td>
</tr>
<tr>
<td>Control</td>
<td>6.75±0.72</td>
<td>9.74±0.96</td>
<td>0.98±0.11</td>
<td>0.96±0.12</td>
</tr>
</tbody>
</table>

Glycogen levels are expressed in terms of µmoles glucose equivalents/gm wet weight/hr.

Each figure is the mean ± the standard error of 6 observations.

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
GLUCOSE UPTAKE, LACTATE AND PYRUVATE PRODUCTION, AND GLYCOGEN SYNTHESIS BY HEMIDIAPHRAGMS PREPARED FROM ALLOXAN-DIABETIC, FASTED RATS.

<table>
<thead>
<tr>
<th></th>
<th>Glucose Uptake (μmoles/gm wet weight/hr)</th>
<th>Lactate Production</th>
<th>Pyruvate Production</th>
<th>Glycogen Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.21±0.78</td>
<td>10.17±1.32</td>
<td>1.14±0.12</td>
<td>2.33±0.42</td>
</tr>
<tr>
<td>Wy 23675 20μM</td>
<td>6.31±0.67</td>
<td>9.71±0.92</td>
<td>0.92±0.10</td>
<td>2.73±0.36</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+1.10</td>
<td>-0.46</td>
<td>-0.22</td>
<td>+0.40</td>
</tr>
<tr>
<td>Control</td>
<td>4.19±0.54</td>
<td>7.99±0.94</td>
<td>0.98±0.08</td>
<td>2.56±0.37</td>
</tr>
<tr>
<td>Wy 23675 200μM</td>
<td>7.76±0.82</td>
<td>8.44±0.91</td>
<td>0.96±0.09</td>
<td>3.92±0.33</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+3.57**</td>
<td>+0.45</td>
<td>-0.02</td>
<td>+1.36*</td>
</tr>
<tr>
<td>Control</td>
<td>3.37±0.50</td>
<td>5.96±0.72</td>
<td>0.76±0.08</td>
<td>1.69±0.29</td>
</tr>
<tr>
<td>Wy 23675 2mM</td>
<td>7.21±0.84</td>
<td>5.03±0.62</td>
<td>0.64±0.07</td>
<td>3.19±0.30</td>
</tr>
<tr>
<td>Difference relative to Control</td>
<td>+3.84**</td>
<td>-0.93</td>
<td>-0.12</td>
<td>+1.50**</td>
</tr>
</tbody>
</table>

Glycogen levels are expressed in terms of μmoles glucose equivalents/gm wet weight/hr.

Each figure is the mean ± the standard error of 6 observations.

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
In chapter 2, it was shown that insulin, tolbutamide, phenformin and Wy 23675 were all able to stimulate glucose utilization \textit{in vivo}. However, tolbutamide did so by stimulating the release of insulin and did not appear to have a direct effect on glucose utilization. Phenformin stimulated glucose utilization without causing the release of insulin but was only able to do this in the alloxan-diabetic rat where blood glucose levels were markedly elevated. Wy 23675, on the other hand, was able to stimulate glucose utilization in both the normal and alloxan-diabetic rat, independent of any effect on insulin release. The results shown in this chapter with rat hemidiaphragms support the above \textit{in vivo} findings on glucose utilization.

Insulin was shown to stimulate glucose uptake and glycogen synthesis into both the normal and alloxan-diabetic rat hemidiaphragm preparations. No significant changes were observed with regard to lactate and pyruvate release. These results for insulin agree well with those of other workers (1-10, 27). The release of alanine and CO$_2$ were not measured in these studies. However, the increased uptake of glucose could not be solely accounted for by the measured increase in glycogen synthesis, so it seems likely that insulin also caused an increase in glucose oxidation and protein synthesis. Other workers have shown that insulin increases glucose oxidation (16, 18, 30) and stimulates the synthesis of protein from amino acids (31-34).

Tolbutamide had no effect on any of the parameters measured in either the normal or alloxan-diabetic rat hemidiaphragms. These results are in agreement with the findings in chapter 2 where tolbutamide had no effect on glucose uptake \textit{in vivo} unless it could stimulate insulin release. The possibility that tolbutamide administered \textit{in vivo} might potentiate the action of insulin on the muscle \textit{in vitro} (25) has not been investigated in this work.
a decrease in glycogen formation in both normal and alloxan-diabetic rat hemidiaphragms. This is in comparison with its effects in vivo where it only stimulated glucose utilization in the alloxan-diabetic rat. However, the 200 μM concentration of phenformin necessary to see these effects in vitro with normal rat hemidiaphragm is considerably higher than the normal plasma level of phenformin seen in patients taking therapeutic doses of the drug [eg: 1-10μM (35)]. In the alloxan rat hemidiaphragm, phenformin had a significant effect on glucose uptake at the concentration of 20μM - a ten-fold lower concentration than that at which a significant effect occurs on glucose uptake in the normal rat hemidiaphragm. Frayn and Adnitt (7) have also shown that hemidiaphragms are more sensitive to the action of phenformin when prepared from alloxan-diabetic rats as opposed to normal rats. Williams and coworkers (12) have also shown an increase in glucose uptake into rat hemidiaphragms caused by phenformin: they showed a similar increase in lactate production and a decrease in glycogen storage with no effect on glucose oxidation.

In muscle from a diabetic animal, the membrane transport of glucose is reduced when compared with normal muscle and this defect is not fully overcome by the addition of insulin in vitro (36-39). Under these conditions, free intracellular glucose accumulates (37,40,41) showing that the phosphorylation of glucose by hexokinase is also impaired and may become the rate-limiting step in glucose utilization in muscle from a diabetic animal. Das (42) has shown that the depression of hexokinase activity in diabetes is due to a decrease in the total activity of this enzyme, as well as to its regulation by the intracellular concentration of glucose-6-phosphate (43). Frayn and Adnitt (7) have proposed that the abnormal free fatty acid metabolism associated with diabetes (44,46) may be involved in the inhibition of hexokinase by virtue of increasing the concentration of glucose-6-phosphate. The elevation of intracellular acetyl CoA concentration caused by fatty acid oxidation leads, via an increase in citrate levels, to inhibition of phosphofructokinase and
diabetic rats hemidiaphragms where phosphorylation is rate-limiting, by causing an increase in the rate of utilization of glucose-6-phosphate in the presence of insulin (7). They found that metformin, in the presence of insulin, increased the glycogen content of hemidiaphragms, which would result in an increased rate of removal of glucose-6-phosphate, and could thus account for the effect of metformin on glucose utilization (7). In contrast, Williams and coworkers (12) showed a decrease in the glycogen content of the rat hemidiaphragm in the presence of phenformin and similar effects have been demonstrated in the results reported in this chapter. These findings combined with the measured increase in glucose uptake make the proposed mechanism of action of phenformin on glucose utilization as put forward by Frayn and Adnitt (7) seem unlikely.

Other workers have failed to show any effects of biguanides on glucose uptake into muscle in either the presence or absence of insulin (21, 22, 26). Kemmer and coworkers (22) found a decreased rate of glycolytic flux together with an unaffected rate of glucose uptake in normal rats treated with metformin, despite a demonstrable hypoglycaemic effect of metformin in vivo. They and other workers (22, 47, 48), in agreement with the findings of Frayn and Adnitt (7), who, however, demonstrated an increase in glucose uptake, showed an increase in glycogen synthesis to account for what happened to glucose under these conditions. Kemmer and coworkers (22) also showed an inhibition of lactate oxidation in the presence of added insulin. Inhibition of pyruvate oxidation by biguanides has been demonstrated by other researchers (49, 50). Kemmer and coworkers (22) suggest, on the basis of these results, that biguanides inhibit muscle pyruvate dehydrogenase (the activity of pyruvate carboxylase is virtually zero in skeletal and heart muscle (51)), and that this action could account for the development of lactic acidosis, which has been repeatedly associated with the use of biguanides (52-57). However, the extent to which
The apparent disagreements about the effects of the biguanides on muscle carbohydrate metabolism may be due to the different experimental conditions and different concentrations of biguanide used. The results reported in this chapter appear to be in agreement with some workers and in disagreement with others.

The occurrence of an increase in glucose uptake together with a decrease in hemidiaphragm glycogen content and an increase in lactate production could best be explained by an increase in glycolysis together with a possible inhibition of lactate oxidation. There was a slight rise in pyruvate production, but this was not statistically significant (Student's t-Test). However, an approximate 10:1 ratio of lactate and pyruvate was maintained when a significant increase in lactate production was measured, indicating a possible similar decrease in pyruvate oxidation in agreement with the findings of other workers (22, 49, 50).

Wy 23675 stimulated glucose uptake and glycogen synthesis, while having no effect on lactate or pyruvate production, in both normal and alloxan-diabetic rat hemidiaphragms. The concentrations at which these effects were observed were similar in both normal and alloxan-diabetic rat hemidiaphragms - 200μM. (Plasma concentrations of Wy 23675 in the region of 160μM have been measured in monkeys following an oral hypoglycaemic dose of the drug (see Chapter 1).) In hemidiaphragms from both normal and alloxan-diabetic rats the increase in glucose uptake was approximately 100% and the increase in glycogen content about 80%. The measured increase in glucose uptake could not be solely accounted for by an increase in glycogen synthesis and, as there was no increase in lactate or pyruvate production, the results suggest an increase in glucose oxidation. However, no measurements were made of oxygen consumption or of carbon dioxide production by hemidiaphragms in these experiments.
uptake and glycogen synthesis to those of insulin, but differ from those of phenformin with regard to glycogen synthesis and lactate production. Insulin has been shown to activate pyruvate dehydrogenase in alloxan-diabetic heart muscle (60); as there was no increase in lactate production in the presence of Wy 23675, it is possible that this enzyme is also activated by Wy 23675.
References.

Chapter 4
THE EFFECTS OF INSULIN, TOLBUTAMIDE, PHENFORMIN AND WY 23675 ON GLUCOSE INCORPORATION INTO ADIPOCYTE LIPIDS.

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This chapter contains the results of experiments carried out with isolated epididymal adipocytes (prepared from both normal and alloxan-diabetic rats) to investigate the effects of selected hypoglycaemic agents upon glucose incorporation into adipocyte lipids.

Results of incubations carried out with adipocytes isolated from normal, fasted rat epididymal fat pads showed the following effects of these agents:-

1. Insulin stimulated glucose uptake into adipocyte lipids in a dose-related manner to a maximum of approximately 260% of control values.

2. Tolbutamide had no effect on basal incorporation of glucose into adipocyte lipids at therapeutic dose levels (approx. 10mM), but did cause a slight stimulation (to 125% of control values) at higher concentrations. Further experiments showed that tolbutamide in high concentrations could potentiate a submaximal insulin-stimulation of glucose incorporation, but had no effect on a maximal response to insulin.

3. Phenformin stimulated glucose incorporation into adipocyte lipids (to approximately 200% of control values), but only at concentrations well above those obtained in the blood with therapeutic doses (approximately 1000 x greater concentrations). Phenformin, at these high concentrations, was able to further stimulate a maximal response to insulin.

4. Wy 23675 stimulated glucose incorporation into adipocyte lipids (to approximately 140% of control values) at concentrations obtained in the blood with hypoglycaemic doses (ie: approximately 100μM). Wy 23675 stimulated further a maximal response to insulin, but only at concentrations approximately 100 x those obtained with hypoglycaemic doses of the drug.

Results of incubations carried out with isolated adipocytes prepared from alloxan-diabetic, fasted rats showed the following effects of these agents:-

1. The effect of alloxan-diabetes was to markedly reduce the basal rate
of incorporation of glucose into adipocyte lipids (to approximately 50% of the normal values).

2. Insulin stimulated the incorporation of glucose into adipocyte lipids to a maximum of approximately 190% of alloxan-diabetic control values - this was still well below normal control values.

3. Tolbutamide had no significant effects on the rate of glucose incorporation into adipocyte lipids, even at high concentrations that were stimulatory in normal fat cells.

4. Phenformin had no effect on glucose incorporation into adipocyte lipids except for a stimulatory effect at a concentration approximately 1000x greater than that normally observed with therapeutic doses of the drug.

5. Wy 23675 stimulated glucose incorporation into adipocyte lipids (to approximately 190% of alloxan-diabetic control values) at concentrations obtained in the blood with hypoglycaemic doses (ie: approximately 100µM) - this level of stimulation was still well below normal control values.
Introduction.

The in vivo work described in Chapter 2 has shown that insulin, tolbutamide, phenformin and Wy 23675 have effects on glucose utilization. Two major sites of glucose utilization are fat and muscle; the work in this chapter looks at the incorporation of radiolabelled glucose into the lipids of isolated adipocytes.

Isolated epididymal adipocytes have been used extensively to study lipid metabolism in vitro. Similar results have been obtained with both fat pads (7,8,14) and isolated adipocytes (1,4,5,9,10). The latter have been employed for the work described in this chapter for the advantages they possess over whole fat pads in terms of the numbers of incubations that can be carried out from the same cell pool and the reproducibility of results.

Insulin has been shown by many workers to stimulate the rate of conversion of glucose into fat in rat epididymal adipose tissue (1,4,5,8,16,17). The mechanism of action is still not fully understood, although insulin has been shown to increase glucose transport (7,9,10,13), pyruvate dehydrogenase activity (18-29), acetyl CoA carboxylase activity (30,31), and possibly one or more steps in the process of fatty acid esterification (32,33). Crofford and Renold (7) showed that the transport of glucose from the extracellular space to the intracellular space was the major rate-limiting step in glucose uptake under conditions of adequate diffusion; and that facilitation of glucose transport was the principle site of insulin action. However, insulin can stimulate fatty acid synthesis independent of its effects on glucose transport. Halperin (14) demonstrated an increase in the conversion of pyruvate to fatty acids stimulated by insulin in adipose tissue from both normal and alloxan-diabetic rats.

Little work appears to have been done with regard to the effects of sulphonylureas on glucose utilization by rat adipose tissue, although
there is evidence to suggest that tolbutamide (40) and chlorpropamide (41) inhibit lipolysis.

The biguanides have been more extensively investigated with conflicting results. Some workers have reported that phenformin caused an increase in glucose uptake and utilization by rat adipose tissue (15,43-45), while others have shown that phenformin depressed glucose uptake and utilization (46,47).

The work described in this chapter compares the effects of insulin, tolbutamide and phenformin with those of Wy 23675 on glucose incorporation into the lipids of isolated adipocytes prepared from both normal and alloxan-diabetic rats.
The sources of all drugs used were given in earlier chapters.

\((^3\text{H}-3)\)-glucose was obtained from the Radiochemical Centre, Amersham, Bucks.

The constituents of the toluene-based scintillant were obtained from Koch-Light Laboratories, Colnbrook, Bucks.

Collagenase and Bovine serum albumin were obtained from Sigma, Kingston-upon-Thames, Surrey.

**Methods.**

The method used was based on that of Moody et al. (1) and involved incubating rat epididymal fat pads with collagenase in order to isolate the adipocytes. Glucose incorporation into adipocyte lipids was measured by incubating adipocytes with \((^3\text{H}-3)\)-glucose. Total lipids were extracted by adding a toluene-based scintillant (2) directly to the incubation vials and the radio-activity in the lipids was measured by liquid scintillation counting.

**The Use of \((^3\text{H}-3)\)-glucose to Measure the Incorporation of Glucose into Adipose Tissue.**

Moody et al. (1) have recommended the use of \((^3\text{H}-3)\)-glucose to measure glucose incorporation into adipocyte lipids, based on previous knowledge concerning the fate of \(^3\text{H}\) attached to different carbon atoms of glucose. \((^3\text{H}-3)\)-glucose can form three primary labelled products in adipose tissue (2). These are:

(i). \(^3\text{HOH}\) from the isomerization of dihydroxyacetone-\(P\) with glyceraldehyde-3-\(P\) in the Embden-Meyerhof pathway.

\[
\begin{align*}
\text{H}^+ + \text{OH}^- + ^3\text{H}-\text{OH} & \xrightarrow{\text{isomerase}} \text{Triosephosphate} \\
\text{H}^+ + \text{OH}^- + \text{H}^- & \xrightarrow{\text{isomerase}} ^3\text{HOH} + \text{H}^+ \text{CO}_2
\end{align*}
\]

(ii). NADP\(^3\text{H}\) from the oxidation of 6-phosphogluconic acid in the Pentose phosphate pathway.

\[
\begin{align*}
\text{HO}-\text{H}^- + \text{NADP}^+ \text{Mg}^{++} & \xrightarrow{\text{oxidation}} \text{6-phosphogluconic acid} \\
\text{H}^+ + \text{OH}^- + \text{H}^- & \xrightarrow{\text{oxidation}} \text{3-ketohexonic acid (intermediate)} \\
\text{H}^+ + \text{OH}^- + \text{H}^- & \xrightarrow{\text{oxidation}} \text{Ribulose-5-P} \\
\text{H}^+ + \text{OH}^- + \text{H}^- & \xrightarrow{\text{oxidation}} \text{Ribulose-5-P} \\
\text{H}^+ + \text{OH}^- + \text{H}^- & \xrightarrow{\text{oxidation}} \text{Ribulose-5-P} \\
\end{align*}
\]
\[
\begin{align*}
\text{Dihydroxyacetone-P} & \rightarrow \text{Glycerol-3-P} \\
\text{\( ^*H\text{-C}O\text{-OH}\)} + \text{NADH} + H^+ & \rightarrow \text{Glycerol phosphate} \xrightarrow{\text{Glycerol kinase}} \text{\( ^*H\text{-C}O\text{-OH}\)} + \text{H}^+ + \text{ADP} + \text{Mg}^{++} \\
\text{H}^{-}C-\text{OPO}_3\text{H}_2 & \rightarrow \text{HO-}C-\text{OH} + \text{ATP}
\end{align*}
\]

Also, a significant amount of \(^3H\) appears in fatty acids through reduction via NADP\(^3H\) - there is a very efficient utilization of NADPH for fatty acid reduction (2). No \(^3H\) appears in lactate, which indicates that NADP\(^3H\) does not exchange \(H^+\) with NADH (2).

Preparation of Isolated Adipocytes.

The preparation of isolated adipocytes was based on the methods of Gliemann (4) and of Rodbell (5). 110-140 gm male rats were killed by decapitation and the epididymal fat pads were rapidly removed and rinsed in Krebs-bicarbonate buffer. Each fat pad was cut into four pieces and then placed in a plastic incubation vial containing 1 ml of Krebs-bicarbonate buffer containing 0.1 mg/ml glucose, 35 mg/ml dialyzed bovine serum albumin - fraction V, and 2 mg/ml collagenase type II. Incubations were carried out at 37°C in an atmosphere of 95% O\(_2\)/5% CO\(_2\), shaken at 120 rpm for 1 hr.

At the end of the incubation period the incubations from several rats were pooled in a plastic dish and the fat cells liberated from tissue fragments by gently stirring with a rod. Any visible tissue fragments were removed with forceps. The free cells were then centrifuged in polythene tubes at 400 g for 1 min. The adipocytes floated to the surface and the infranatant was discarded by aspiration. The adipocytes were then resuspended in Krebs-bicarbonate medium containing 10 mg% glucose and 10 mg/ml bovine serum albumin, fraction V. The adipocytes were centrifuged and resuspended a further three times, the final suspension being made up to give 3-5 mgs lipid/ml.
Incubations with \(^3\)H\(^-\)glucose.

1 ml aliquots of the cell suspension were used for the incubations, which were carried out in plastic vials in a water bath set at 37°C and shaking at 120 rpm. 0.5 μCi of \(^3\)H\(^-\)glucose was added to each incubation vial in a 20 μls volume. All drugs were also added in 20 μls of Krebs-bicarbonate medium (at 50x the desired final concentration) to give a total incubation volume of 1.04 mls.

The incubations were terminated by the addition of 15 mls of toluene-based scintillant (5g. Packard Premix or 5g. of 2.5 dipheylloxazol + 0.3g. 1,4-bis-2,2(4-methyl-5-phenoloxazoly1)-benzene in 1 litre of toluene). Lipids were extracted by adding the scintillator and allowing to stand overnight at 4°C before counting. The results were expressed as disintegrations per minute/5 mg lipid (6).
Results.

a) Glucose Incorporation into Lipids of Isolated Adipocytes Prepared from Normal, Fasted Rats.

Insulin caused a time (Fig. 4(i)) and a dose-related (Table 4(i)) increase in the incorporation of \(^3\)H-glucose into the lipids of isolated adipocytes to a maximum of approximately 260% of control values. Concentrations of insulin as low as 5 \(\mu\)Units/ml caused measurable and statistically significant (Student's t-Test) increases in glucose incorporation into lipids. In contrast, tolbutamide appeared to have no effect on glucose incorporation into lipids of adipocytes except at 20mM concentration, where it caused a small (25%) increase (Table 4(i)). Tolbutamide had no effect on the maximal stimulation of glucose incorporation into adipocyte lipids caused by insulin, but it did further enhance the submaximal insulin effect (Table 4(i)) when given in high concentrations (20mM). Phenformin doubled the rate of glucose incorporation into adipocyte lipids, but this only occurred at concentrations (2-20mM; Table 4(i)) a thousand-fold or more greater than those obtained in the plasma with therapeutic administration of the drug (79-82). Phenformin was also able to further stimulate both sub-maximal and maximal insulin effects on glucose incorporation into adipocyte lipids (Table 4(i)) - however, these effects were again only seen at high concentrations. Wy 23675 stimulated glucose incorporation into adipocyte lipids at 0.2mM (Table 4(i)), which is a concentration obtained in monkeys with a hypoglycaemic dose of the drug. Wy 23675 also stimulated both submaximal and maximal insulin effects on glucose incorporation into adipocyte lipids (Table 4(i)) - however, higher concentrations than those that have been obtained in vivo were necessary for this effect to be apparent.

b) Glucose Incorporation into Lipids of Isolated Adipocytes Prepared from Alloxan-Diabetic, Fasted Rats.

Alloxan-diabetes resulted in a marked decrease (to approximately 30%...
of controls) in the rate of incorporation of glucose into the lipids of isolated adipocytes [Fig. 4(ii)]. The mean blood glucose levels of the fasted, alloxan-diabetic rats was 20.76 mmol/l. (± SEM of 7.24) compared with the mean of 3.87 mmol/l. (± SEM of 0.29) in normal rats. Insulin stimulated the incorporation of glucose into lipids to approximately the same extent (about 200% of controls) in adipocytes from alloxan-diabetic rats [Table 4(ii)] as from normal rats [Table 4(i)]. However, as the adipocytes from alloxan-diabetic rats had a measured glucose incorporation into lipid of only 30% of the normal rats, insulin did not totally compensate for the effect of alloxan-diabetes. Tolbutamide, in contrast to its effects in normal rat adipocytes, had no measurable effects on glucose incorporation into alloxan-diabetic rat adipocyte lipids at any of the concentrations used [Table 4(ii)]. Phenformin, as in the normal rat adipocytes, stimulated glucose incorporation into the lipids of alloxan-diabetic rat adipocytes [Table 4(ii)], but again required a higher concentration than that normally obtained with therapeutic use of the drug in order to have its effect (79-82). Wy 23675, as in the normal rat adipocyte, stimulated the incorporation of glucose into the lipids of adipocytes from alloxan-diabetic rats [Table 4(ii)]. Like insulin, the degree of stimulation was approximately 200% of the control values and thus not sufficient to compensate for the effect of alloxan-diabetes.
Chapter 4.

THE EFFECT OF INSULIN, TOLBUTAMIDE, PHENFORMIN AND WY 23675 ON GLUCOSE INCORPORATION INTO THE LIPOIDS OF ISOLATED ADIPOCYTES PREPARED FROM NORMAL AND ALLOXAN-DIABETIC, FASTED RATS.

Figure No. Title
4(i) Effect of Insulin with Time on $({}^3\text{H}-3)$-Glucose Incorporation into Lipids of Isolated Adipocytes Prepared from Normal, Fasted Rats.
4(ii) Comparison of $({}^3\text{H}-3)$-Glucose Uptake into Lipids of Isolated Adipocytes Prepared from Normal and Alloxan-Diabetic Fasted Rats.

Table No. Title
4(i) Glucose Incorporation into Lipids of Isolated Adipocytes Prepared from Normal, Fasted Rats.
4(ii) Glucose Incorporation into Lipids of Isolated Adipocytes Prepared from Alloxan-Diabetic, Fasted Rats.
EFFECT OF INSULIN WITH TIME ON $\left(\text{H}^3\right)$-GLUCOSE INCORPORATION INTO LIPIDS OF
ISOLATED ADIPOCYTES PREPARED FROM NORMAL, FASTED RATS.

Isolated adipocytes were prepared from the epididymal fat pads of 18-hour fasted, normal rats and incubated as described in Methods. Incubations were carried out in the absence (controls – O) or presence of insulin 200 μUnits/ml (△) and were stopped at the time points shown by the addition of a toluene based scintillant. The $^3\text{H}$ in the lipid extract was counted and the results expressed as dpm/5 mg lipid. Each time point represents the mean ± the standard error of 6 observations.

$^3\text{H}$ in Lipid Extract

(10^3 dpm/5 mg lipid)

Time (mins)
GLUCOSE INCORPORATION INTO LIPIDS OF ISOLATED ADIPOCYTES PREPARED FROM NORMAL, FASTED RATS.

Isolated adipocytes were prepared from the epididymal fat pads of 18-hr fasted, normal rats and incubated as described in methods. The $^3$H in the lipid extract was counted and the results expressed as dpm/5 mg lipid. Each value represents the mean ± the standard error of 6 observations. Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* p<0.05 ;  ** p<0.01 ;  *** p<0.001.

1. Effect of Insulin on ($^3$H-3)-glucose Uptake into Isolated Adipocytes over 2 hrs Incubation.

<table>
<thead>
<tr>
<th>Insulin Concentration (mU/ml)</th>
<th>$^3$H in Lipid Extract (dpm/5 mg lipid)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65172 ± 4522</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>72787 ± 5412</td>
<td>110</td>
</tr>
<tr>
<td>5</td>
<td>78493 ± 3247</td>
<td>120*</td>
</tr>
<tr>
<td>10</td>
<td>97106 ± 5423</td>
<td>149**</td>
</tr>
<tr>
<td>50</td>
<td>14162 ± 6219</td>
<td>221***</td>
</tr>
<tr>
<td>100</td>
<td>16537 ± 7498</td>
<td>254***</td>
</tr>
<tr>
<td>200</td>
<td>184322 ± 10127</td>
<td>283***</td>
</tr>
<tr>
<td>400</td>
<td>171758 ± 9876</td>
<td>264***</td>
</tr>
</tbody>
</table>

2. Effect of Tolbutamide on ($^3$H-3)-glucose Uptake into Isolated Adipocytes

<table>
<thead>
<tr>
<th>Tolbutamide Concentration (mM)</th>
<th>$^3$H in Lipid Extract (dpm/5 mg lipid) at the following times (mins): 30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>11794 ± 876</td>
<td>25374 ± 1127</td>
<td>52911 ± 2569</td>
</tr>
<tr>
<td>0.2</td>
<td>11244 ± 723</td>
<td>27431 ± 1239</td>
<td>56197 ± 3478</td>
</tr>
<tr>
<td>% of Control</td>
<td>95%</td>
<td>108%</td>
<td>98%</td>
</tr>
<tr>
<td>2</td>
<td>12762 ± 419</td>
<td>19982 ± 2194</td>
<td>54657 ± 5431</td>
</tr>
<tr>
<td>% of Control</td>
<td>108%</td>
<td>79%</td>
<td>94%</td>
</tr>
<tr>
<td>20</td>
<td>14778 ± 523</td>
<td>31286 ± 1572</td>
<td>65567 ± 3261</td>
</tr>
<tr>
<td>% of Control</td>
<td>125%</td>
<td>123%</td>
<td>113%</td>
</tr>
</tbody>
</table>

3. Effect of Tolbutamide on Sub-maximal and Maximal Insulin-Stimulated ($^3$H-3)-glucose Uptake into Isolated Adipocytes over 2 hrs Incubations.

<table>
<thead>
<tr>
<th>Insulin Concentration (mU/ml)</th>
<th>Tolbutamide Concentration (mM)</th>
<th>$^3$H in Lipid Extract (dpm/5 mg lipid)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>76148 ± 523</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>150773 ± 7874</td>
<td>198</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>159149 ± 12113</td>
<td>209</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>168287 ± 8524</td>
<td>221</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>199065 ± 9733</td>
<td>250*</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>199508 ± 12367</td>
<td>262</td>
</tr>
<tr>
<td>200</td>
<td>0.2</td>
<td>188086 ± 7948</td>
<td>247</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
<td>204838 ± 8631</td>
<td>269</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>208646 ± 6469</td>
<td>274</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>193416 ± 11237</td>
<td>254</td>
</tr>
</tbody>
</table>
4. Effect of Phenformin on (3H-3)-glucose Uptake into Isolated Adipocytes

<table>
<thead>
<tr>
<th>Phenformin Concentration (mM)</th>
<th>3H in Lipid Extract (dpm/5 mg lipid) at the following times (mins):-</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>8976 ± 659</td>
<td>18144 ± 984</td>
<td>39274 ± 2417</td>
<td></td>
</tr>
<tr>
<td>0.2 % of control</td>
<td>10188 ± 712</td>
<td>20321 ± 941</td>
<td>42416 ± 9726</td>
<td>112%</td>
</tr>
<tr>
<td>2 % of control</td>
<td>16965 ± 894</td>
<td>35199 ± 2149</td>
<td>65588 ± 5124</td>
<td>189%***</td>
</tr>
<tr>
<td>20 % of control</td>
<td>18311 ± 1072</td>
<td>56469 ± 2432</td>
<td>84046 ± 5174</td>
<td>204%***</td>
</tr>
</tbody>
</table>

5. Effect of Phenformin on Sub-maximal and Maximal Insulin-Stimulated (3H-3)-glucose Uptake into Isolated Adipocytes over 2 hrs Incubations.

<table>
<thead>
<tr>
<th>Insulin Concentration (uUnits/ml)</th>
<th>Phenformin Concentration (mM)</th>
<th>3H in Lipid Extract (dpm/5 mg lipid)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>61342 ± 3274</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>101214 ± 6273</td>
<td>165</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>97534 ± 5924</td>
<td>159</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>126978 ± 5421</td>
<td>207*</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>139246 ± 8262</td>
<td>227**</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>171144 ± 12977</td>
<td>279</td>
</tr>
<tr>
<td>200</td>
<td>0.2</td>
<td>175438 ± 9742</td>
<td>286</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
<td>194454 ± 14127</td>
<td>317</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>223285 ± 11722</td>
<td>364*</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>172984 ± 10999</td>
<td>282</td>
</tr>
</tbody>
</table>

6. Effect of Wy 23675 on (3H-3)-glucose Uptake into Isolated Adipocytes

<table>
<thead>
<tr>
<th>Wy 23675 Concentration (mM)</th>
<th>3H in Lipid Extract (dpm/5 mg lipid) at the following times (mins):-</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>11926 ± 897</td>
<td>23641 ± 1021</td>
<td>44237 ± 2229</td>
<td></td>
</tr>
<tr>
<td>0.2 % of control</td>
<td>16935 ± 924</td>
<td>32388 ± 1298</td>
<td>66798 ± 2747</td>
<td>142%**</td>
</tr>
<tr>
<td>2 % of control</td>
<td>31604 ± 1262</td>
<td>64304 ± 2721</td>
<td>116343 ± 6684</td>
<td>292%**</td>
</tr>
<tr>
<td>20 % of control</td>
<td>34824 ± 2149</td>
<td>72342 ± 3516</td>
<td>126960 ± 5172</td>
<td>292%**</td>
</tr>
</tbody>
</table>

155
7. Effect of Wy 23675 on Sub-maximal and Maximal Insulin-Stimulated \((\text{\textsuperscript{3}}\text{H})\)-glucose Uptake into Isolated Adipocytes over 2 hrs Incubations.

<table>
<thead>
<tr>
<th>Insulin Concentration (\text{\textmu}Units/ml)</th>
<th>Wy 23675 Concentration (mM)</th>
<th>\text{\textsuperscript{3}}\text{H} in Lipid Extract (dpm/5 mg lipid)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>51376 ± 3100</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>94532 ± 6274</td>
<td>184</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>110976 ± 5156</td>
<td>216</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>142311 ± 8970</td>
<td>277**</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>162348 ± 9443</td>
<td>316***</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>144880 ± 10721</td>
<td>282</td>
</tr>
<tr>
<td>200</td>
<td>0.2</td>
<td>152587 ± 7479</td>
<td>297</td>
</tr>
<tr>
<td>200</td>
<td>2</td>
<td>167486 ± 8867</td>
<td>326</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>202421 ± 10217</td>
<td>394**</td>
</tr>
<tr>
<td>400</td>
<td>0</td>
<td>139229 ± 9766</td>
<td>271</td>
</tr>
</tbody>
</table>

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* \( p \leq 0.05 \) ; ** \( p \leq 0.01 \) ; *** \( p \leq 0.001 \)
Isolated adipocytes were prepared from the epididymal fat pads of 18 hr fasted rats. Control rats were previously untreated, whilst alloxan-diabetic rats were treated with alloxan (0.7 mmol/kg i.p.) two days previously and those with blood glucose levels in excess of 16.5 mmol/l were used. Incubations were carried out as described in Methods with fat cells from either control (■) or alloxan-diabetic (●) rats. Each point on the figure represents the mean ± the standard error of 6 observations.
GLUCOSE INCORPORATION INTO LIPIDS OF ISOLATED ADIPOCYTES PREPARED FROM ALLOXAN-DIABETIC, FASTED RATS.

Isolated adipocytes were prepared from the epididymal fat pads of 18-hr. fasted alloxan-diabetic rats and incubated as described in Methods. The $^3$H in the lipid extract was counted and expressed as dpm/5 mg lipid. Each value represents the mean ± the standard error of 6 observations. Statistically significant changes from the appropriate control were determined by Student's t-Test:

* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

1. Effect of Insulin on ($^3$H-3)-glucose Uptake into Isolated Adipocytes over 2 hrs Incubation.

<table>
<thead>
<tr>
<th>Insulin Concentration (μUnits/ml)</th>
<th>$^3$H in Lipid Extract (dpm/5 mg lipid)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25263 ± 2796</td>
<td>100%</td>
</tr>
<tr>
<td>2.5</td>
<td>24253 ± 2318</td>
<td>96%</td>
</tr>
<tr>
<td>5</td>
<td>28800 ± 3134</td>
<td>114%</td>
</tr>
<tr>
<td>10</td>
<td>34863 ± 3666</td>
<td>138%</td>
</tr>
<tr>
<td>50</td>
<td>41431 ± 3978</td>
<td>164%**</td>
</tr>
<tr>
<td>100</td>
<td>46231 ± 4868</td>
<td>183%**</td>
</tr>
<tr>
<td>200</td>
<td>49768 ± 4627</td>
<td>197%**</td>
</tr>
<tr>
<td>400</td>
<td>45726 ± 5012</td>
<td>181%**</td>
</tr>
</tbody>
</table>

2. Effect of Tolbutamide on ($^3$H-3)-glucose Uptake into Isolated Adipocytes

<table>
<thead>
<tr>
<th>Tolbutamide Concentration (mM)</th>
<th>$^3$H in Lipid Extract (dpm/5 mg lipid) at the following times (mins) :-</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>3921 ± 407</td>
<td>100%</td>
</tr>
<tr>
<td>0.2</td>
<td>3294 ± 398</td>
<td>84%</td>
</tr>
<tr>
<td>% of Control</td>
<td>112%</td>
<td>98%</td>
</tr>
<tr>
<td>2</td>
<td>4592 ± 464</td>
<td>112%</td>
</tr>
<tr>
<td>% of Control</td>
<td>109%</td>
<td>116%</td>
</tr>
<tr>
<td>20</td>
<td>4274 ± 447</td>
<td>109%</td>
</tr>
</tbody>
</table>

3. Effect of Phenformin on ($^3$H-3)-glucose Uptake into Isolated Adipocytes

<table>
<thead>
<tr>
<th>Phenformin Concentration (μM)</th>
<th>$^3$H in Lipid Extract (dpm/5 mg lipid) at the following times (mins) :-</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>6766 ± 873</td>
<td>100%</td>
</tr>
<tr>
<td>20</td>
<td>7510 ± 866</td>
<td>111%</td>
</tr>
<tr>
<td>% of Control</td>
<td>110%</td>
<td>109%</td>
</tr>
<tr>
<td>200</td>
<td>6225 ± 734</td>
<td>92%</td>
</tr>
<tr>
<td>% of Control</td>
<td>105%</td>
<td>87%</td>
</tr>
<tr>
<td>2000</td>
<td>11029 ± 1432</td>
<td>92%</td>
</tr>
<tr>
<td>% of Control</td>
<td>163%*</td>
<td>178%*</td>
</tr>
</tbody>
</table>

Each figure is the mean ± the standard error of 6 observations.
### Effect of Wy 23675 on \(^3\)H-3-glucose Uptake into Isolated Adipocytes

<table>
<thead>
<tr>
<th>Wy 23675 Concentration ((\mu M))</th>
<th>(^3)H in Lipid Extract (dpm/5 mg lipid) at the following times (mins):</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (control)</td>
<td>30</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>20 % of Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 % of Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000 % of Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
<td>3286 ± 388</td>
<td>6713 ± 792</td>
<td>12327 ± 2372</td>
<td></td>
</tr>
<tr>
<td>20 % of Control</td>
<td>3976 ± 417</td>
<td>8593 ± 912</td>
<td>14546 ± 1676</td>
<td></td>
</tr>
<tr>
<td>200 % of Control</td>
<td>6112 ± 721</td>
<td>13023 ± 976</td>
<td>23298 ± 2572</td>
<td></td>
</tr>
<tr>
<td>2000 % of Control</td>
<td>6539 ± 687</td>
<td>15037 ± 1621</td>
<td>26626 ± 2922</td>
<td></td>
</tr>
</tbody>
</table>

Each figure is the mean ± the standard error of 6 observations.

Statistically significant changes from the appropriate controls were determined by Student's t-test:

* \(p<0.05\) ; ** \(p<0.01\) ; *** \(p<0.001\)
Discussion.

Insulin, tolbutamide, phenformin and Wy 23675 were all shown to stimulate glucose utilization in vivo (Chapter 2), although tolbutamide could only do so under conditions where it was able to stimulate insulin release. In the previous chapter, insulin, phenformin and Wy 23675 were shown to stimulate glucose uptake into rat hemi-diaphragms, while tolbutamide had no effect on this in vitro measurement. The effects of these agents on glucose utilization have been further investigated in the work described in this chapter with isolated adipocytes.

Insulin was shown to increase the uptake of glucose into the lipids of adipocytes prepared from both normal and alloxan-diabetic rats (Tables 4(i) and 4(ii)). This effect of insulin has been demonstrated by many other researchers (1, 4, 16, 17, 48-50).

The major rate-limiting step in glucose uptake under conditions of adequate diffusion is the transport of glucose from the extracellular space to the intracellular space and it has been proposed that the principle site of insulin action is the facilitation of glucose transport (7, 52-55). However, the fundamental mechanism by which insulin enhances glucose transport is still unknown. Addition of insulin to adipose tissue results in a fall in the levels of cyclic adenosine monophosphate (cAMP) (10), but there is no good evidence that this is directly associated with glucose transport. Taylor and coworkers (10) have shown that when cAMP levels were lowered with antilipolytic agents (eg: insulin, nicotinate, clofibrate), the rates of glucose transport were increased.

Lipolytic agents (eg: glucagon, adrenocorticotropic hormone, isoproterenol, theophylline) elevated cAMP levels and reduced the rate of glucose transport. It has been proposed that cAMP could induce inhibition of glucose transport into adipocytes by the phosphorylation of membrane proteins using ATP as the substrate (10, 13, 51). However, other groups found that under certain conditions insulin had no effect (34-37) or increased (38) cAMP levels in adipocytes, while inhibiting lipolysis. In addition, lipolytic hormones have been shown to increase glucose
indicating that glucose transport can be increased in response to other stimuli even when cAMP levels are elevated. Thus, the importance of the effects of these agents on cAMP levels with respect to their effects on glucose transport remains unclear.

More recently, Olefsky (56) has further investigated the mechanisms by which insulin activates the glucose transport system in rat adipocytes. He used 2-deoxyglucose to measure glucose uptake as 2-deoxyglucose is transported and phosphorylated by the same process as D-glucose, but cannot be further metabolized (57). Olefsky's work (56) indicated that insulin increases the number of carriers available for glucose transport, rather than enhancing the intrinsic activity of already functioning carriers.

Insulin has been reported to increase fatty acid synthesis from glucose in rat epididymal adipose tissue by more than twice the amount it can increase pyruvate output (58-61). This preferential incorporation of pyruvate carbon atoms into fatty acids compared with its release from fat pads in the presence of insulin led to the suggestion that insulin might specifically activate the conversion of pyruvate carbon atoms into fatty acids in addition to stimulating glucose transport. From Taylor and Jungas work (29), it appears that the activity of pyruvate dehydrogenase is one factor limiting the capacity of the pathway controlling the conversion of glucose to fatty acid. It has been shown that insulin activates adipose tissue pyruvate dehydrogenase by means of the phosphorylation-dephosphorylation cycle (the unphosphorylated form being the active form of the enzyme) (18,20,22,29,62), previously shown to influence the activity of the enzyme complex from heart, liver, kidney and brain (63-65). Neither the pyruvate dehydrogenase kinase nor the pyruvate dehydrogenase phosphate phosphatase appear to be directly influenced by cAMP or cGMP (18,66-68), so it is unlikely that these nucleotides could directly mediate the action of insulin on pyruvate dehydrogenase. However, insulin has been shown to activate pyruvate dehydrogenase phosphate phosphatase (69,70) and it is
fraction of pyruvate dehydrogenase present in the tissue in the active unphosphorylated form (70).

Insulin has also been shown to stimulate glycogen synthesis from glucose independent of its effects on glucose transport into adipocytes (55) and to stimulate the oxidation of glucose to carbon dioxide (4, 5, 8). Insulin stimulates glycogen synthetase I activity (55, 71-73) without changing the total synthase activity (55). Insulin had no effect on phosphorylase a activity, although it did markedly oppose adrenaline-stimulation of phosphorylase a, as well as adrenaline-inhibition of glycogen synthase I (55). However, these aspects of the effect of insulin on glucose utilization by adipocytes are outside the context of the work reported in this chapter.

The sulphonylurea, tolbutamide, was shown to have no marked effects on basal rates of glucose incorporation into adipocytes from either normal or alloxan-diabetic rats, but it did potentiate a submaximal insulin effect, although a high concentration (20 mM) of the drug had to be present for the effect to be recorded (Tables 4(i) and 4(ii)). There is no published data illustrating this effect of sulphonylureas in adipocytes, although there is some evidence to suggest that tolbutamide, administered in vivo, potentiated the in vitro insulin-stimulation of glucose uptake into skeletal muscle (74). However, in view of the high concentration of tolbutamide required for the observed effect on adipocytes, it is unlikely that this has any physiological significance.

The biguanide, phenformin, stimulated glucose incorporation into adipocyte lipids, but only at very high concentrations (2 mM) (Tables 4(i) and 4(ii)). Tranquada and Beigelman (15) have reported a stimulatory effect on glucose uptake into fat pads of phenformin at a concentration of 20 \mu M with no effect on lactate production. This contrasts with the findings of Pereira et al (47), who found that phenformin inhibited glucose oxidation to CO₂, inhibited lipogenesis and caused lactate accumulation. However, Pereira et al (47) used 0.5 mM phenformin in their experiments, which is a concentration that has been shown to block
greater than concentrations of phenformin normally obtained in the plasma with therapeutic doses of the drug (79-82). The findings reported in this chapter show no evidence of any inhibitory effects of phenformin on lipogenesis, but, in contrast to Pereira et al. (47), show a stimulation at high concentrations. However, the concentrations at which these effects occurred were too high to have any therapeutic significance. The low doses of phenformin (20μM) at which Tranquada and Beigelman (15) showed stimulatory effects on glucose uptake have not affected glucose incorporation into lipid in the work reported in this chapter. Similarly, the findings of other workers (78), who, in contrast to Tranquada and Beigelman, showed inhibitory effects of biguanides on lipogenesis in concentrations similar to those seen in the plasma with therapeutic administration of the drug, have not been confirmed. Thus, with the conflicting results reported in the literature and the high concentrations of phenformin required for the results reported in this chapter, it is difficult to analyze biguanide action on lipogenesis and its importance in terms of effects on glucose utilization in vivo.

Wy 23675 stimulated the incorporation of glucose into adipocyte lipids in concentrations obtained in the blood with hypoglycaemic doses of the drug [Tables 4(i) and 4(ii)]; and the results reported in Chapter 3 demonstrated that Wy 23675 increased glucose utilization by rat hemidiaphragm. Taken together, these results illustrate an effect of Wy 23675 on glucose utilization by peripheral tissues in vitro, which has been shown in vivo using radiolabelled glucose (see Chapter 2).

Like insulin, Wy 23675 stimulated lipogenesis from glucose; however, further work is required in order to determine the mechanism of action of Wy 23675. The results reported in Chapter 3 indicate that Wy 23675 could possibly activate pyruvate dehydrogenase. Insulin has been shown to activate this enzyme in adipose tissue (18,20,22,29,62) and, as this may be one of the rate-limiting factors in the conversion of glucose to fatty acid (29), this could be a significant site of action for both insulin and Wy 23675.
References:

72. Steinberg, D., Mayer, S. E., Khoo, J. C., Miller, E. A., Fredholm, B. & Eichner, R.


Chapter 5.

THE EFFECTS OF INSULIN, TOLBUTAMIDE, PHENFORMIN AND WY 23675 ON GLUCOSE PRODUCTION BY RAT LIVER.

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   (vii) The effects of insulin, tolbutamide, phenformin and Wy 23675 on gluconeogenesis in alloxan-diabetic rats .......... 221
7. References ............................................................. 223
The figures in this section show the results of experiments, which investigate the effects of selected hypoglycaemic agents on glucose production by the rat liver. Initially, perfused livers were used and the results correlated with similar experiments with isolated hepatocytes, which were used thereafter.

Results from perfusions with livers from 18-hr fasted rats showed the following effects of these agents:

1. The rate of gluconeogenesis from alanine was markedly less than that from lactate plus pyruvate.
2. Glucagon trebled gluconeogenesis from both alanine and lactate plus pyruvate.
3. Insulin had no significant effect on gluconeogenesis from lactate plus pyruvate.
4. Both phenformin and Wy 23675 inhibited gluconeogenesis from lactate plus pyruvate.

Results from isolated liver cells prepared from fed rats showed the following effects of these agents:

1. Glucagon more than doubled the basal rate of glucose production.
2. Insulin had no effect on basal rates of glucose production, but reduced glucagon-stimulation of it.
3. No effect was observed on either basal or glucagon-stimulated glucose production, by tolbutamide, phenformin, or Wy 23675.

Results from isolated hepatocytes prepared from 18-hr fasted rats showed the following effects of these agents:

1. The relative rates of gluconeogenesis from selected substrates were:
   xylitol > dihydroxyacetone > lactate plus pyruvate > alanine.
2. Glucagon stimulated gluconeogenesis from alanine, lactate plus pyruvate and dihydroxyacetone, but not from xylitol.
3. Insulin had no effect on gluconeogenesis from lactate plus pyruvate, but did reduce glucagon-stimulation of it.
4. Tolbutamide had no effect on either gluconeogenesis from lactate plus pyruvate or on glucagon-stimulation of it.

5. Phenformin reduced gluconeogenesis from both lactate plus pyruvate and dihydroxyacetone, but did not affect gluconeogenesis from xylitol.

6. Wy 23675, like phenformin, reduced gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but did not affect gluconeogenesis from xylitol.

Results from isolated hepatocytes prepared from 18-hr fasted, alloxan-diabetic rats showed the following effects of these agents:

1. Alloxan-diabetes resulted in an increase in the rates of gluconeogenesis from lactate plus pyruvate, from dihydroxyacetone and from xylitol compared with control rates from normal rats.

2. Glucagon in vitro stimulated gluconeogenesis from lactate plus pyruvate, but to a markedly lesser extent than in normal control rats.

3. Neither insulin nor tolbutamide in vitro had any effect on gluconeogenesis from lactate plus pyruvate.

4. Insulin administered in vivo inhibited gluconeogenesis in isolated liver cells from lactate plus pyruvate, from dihydroxyacetone and from xylitol.

5. Tolbutamide administered in vivo had no effect on gluconeogenesis from lactate plus pyruvate.

6. Both phenformin and Wy 23675 in vitro inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but had no effect on gluconeogenesis from xylitol.
Introduction.

In Chapter 2, the effects of insulin, tolbutamide, phenformin and WY 23675 upon glucose utilization in vivo were studied. The use of \(^{3}H\)-glucose in these experiments meant that the effects of these agents on glucose utilization were observed distinct from any effects that they also may have had on glucose synthesis. The work reported in this chapter looks at the effects of these agents on glucose production by the rat liver.

The rat liver has been used extensively in biochemical studies, particularly with regard to various aspects of glucose metabolism (23-35). The isolated perfused liver has been used in many of these studies, although there has been a considerable variation in the techniques used to perfuse the organ (1-5). The perfused liver provides a good technique for continuous monitoring of the products of liver metabolism. The main disadvantage is that the time for a single response can be very long in relation to the limited useful life of the preparation, so that very few results can be obtained per liver. This inevitably means that a great deal of repetition is necessary to establish a response.

Isolated hepatocytes have been used more recently in investigations into liver metabolism (6-22). Their chief advantage lies in the fact that many results can be obtained from a single liver. Early doubts about their ability to retain their metabolic integrity have been largely allayed. Hems et al (2) have suggested that a stringent test of metabolic integrity of hepatocytes is the ability to produce glucose from lactate since this process requires energy as well as the cooperation and integrity of both the mitochondrial and cytoplasmic compartments; isolated hepatocytes have been shown to have this capability (18, 20).

Results from work with isolated hepatocytes have been comparable to those from perfused livers. Ingebretson and Wagle (20) made a direct comparison on glucose formation by each of these preparations from lactate, alanine and pyruvate. They found that the results from the isolated hepatocytes were equivalent to those from the perfused liver;
Garrison and Haynes (21) have produced similar results. The effects of hormones on gluconeogenesis and glycogenolysis have also been looked at in isolated hepatocytes. Garrison and Haynes (21) have shown that glucagon, adrenaline and cyclic AMP stimulate gluconeogenesis from lactate, alanine and pyruvate in fasted isolated hepatocytes. In the perfused liver, Exton et al (23) have shown similar effects. Glucagon, adrenaline and cyclic AMP have been shown to stimulate glycogenolysis in isolated hepatocytes (21, 22), while insulin has been shown to inhibit this effect (22); similar results have been obtained in the perfused liver (33). Glucagon has also been shown to increase cyclic AMP levels in isolated hepatocytes from both fed and fasted rats and this was followed by an increase in glycogenolysis or gluconeogenesis from lactate (21); similar results have been obtained in perfused livers (33).

These findings suggest that the carbohydrate metabolism of isolated hepatocytes closely resembles that of the intact perfused liver. However, to justify the use of isolated hepatocytes in the work reported in this chapter, the initial work on glucose production by the rat liver has been completed in the perfused liver, so that a direct comparison can be made of the two techniques with the drugs being studied.
Materials.

The sources of all drugs other than glucagon were given in earlier chapters. Glucagon, bovine serum albumin, collagenase, hyaluronidase, EGTA, HEPES, lactate, pyruvate, alanine, dihydroxyacetone and xylitol were all obtained from Sigma, Kingston-upon-Thames, Surrey.

Methods.

LIVER PERFUSIONS.

The method used was based on that of Hems et al (2) and enabled the liver to be perfused in situ, although isolated from the rest of the animal.

1). Apparatus.

The perfusion apparatus was based on the designs of Hems et al (2) and was housed in a cabinet with a Perspex door and heated by thermostatically controlled bars with air circulated by a fan to maintain the temperature at 37°C.

2). Perfusion Medium.

The perfusion medium consisted of Krebs bicarbonate medium, dialysed bovine serum albumin Fraction V and washed goat red blood cells obtained fresh from the animal unit at Surrey University. The medium was prepared 18 hrs prior to the liver perfusion, each perfusion requiring 60 mls of medium.

Bovine serum albumin - fatty acid poor - was added to the Krebs bicarbonate medium to give a 3% solution. The pH of the medium was then adjusted to 7.4 with NaOH, since albumin is acidic. The washed red cells were then added to the medium to give a final haematocrit value of approximately 35 and the pH was again checked to see that the value was 7.4.

3). Operative Procedure.

All the experiments with liver perfusions were carried out at Surrey University, using 110-140 gm male rats bred in their animal unit.
The rat was anaesthetized with Nembutal (0.15 mls of a 6% solution per 100 gm. body weight) and the abdomen was opened with a mid-line incision and transverse incisions to the left and right of the mid-line. The rat was then heparinized by injection of 0.1 mls of Pularin into the spleen. The intestines were placed on damp tissue to the animal's left to expose the liver, portal vein, inferior vena cava, and bile duct (see Fig 5(ii)).

A loose ligature was placed around the vena cava above the right renal vein. Next, the bile duct was cannulated with a length of Portex tubing (for intravenous cannulation - 2FG O/D 0.63 mm.). The portal vein was then cannulated with heparinized Portex tubing (5 FG O/D 1.65 mm.) and tied off behind the point of cannulation.

The thorax was then opened by a transverse incision above and along the line of the diaphragm and by two longitudinal incisions towards the head from the ends of the transverse incision. The chest wall was flapped back towards the head and held in position there. The vagus and phrenic nerves and the oesophagus were cut in order to paralyse the diaphragm and eliminate possible vasomotor effects of the vagus. The inferior vena cava was then cannulated with a length of heparinized Portex tubing (5 FG O/D 1.65 mm.) cut to a sharp point and pressed through the right atrium and down the vein towards the diaphragm, where it was tied in place. The loose ligature around the abdominal vena cava was then tied.

The liver was flushed out with 10 mls of the prepared perfusion medium through the cannula in the portal vein, before being attached to the perfusion apparatus as in Fig. 5(i). Care was taken to avoid letting air enter the portal vein during this procedure. The abdomen was covered by a damp tissue on a wire frame to prevent it from drying out during the experiment.

The perfusion pressure was 20-30 cm water obtained by adjusting the height of the reservoir at the base of the bulb oxygenator above the animal platform. The perfusion medium was kept oxygenated by a flow of 95% O₂/5% CO₂ passing over it as it flowed through the bulb oxygenator.
The rate of flow of the medium through the liver was 15-25 ml/min, remaining constant during the experimental period, which did not exceed 140 mins.

Several factors were observed during the course of an experiment as indicators of a successful liver perfusion. These were the maintenance of a uniform red colour of the perfused liver, the maintenance of oxygen uptake from the perfusate as indicated by a change in colour as it passed through the liver, and the maintenance of a flow of bile from the cannula in the bile duct. Preparations that failed to satisfy these requirements were discarded. Each liver was weighed at the end of the experiment, so that the results could be expressed in terms of gms. wet liver weight.

4). Perfusate Sampling and Addition of Substrates and Drugs.

Perfusate samples were taken from the reservoir below the animal platform at intervals throughout the experiment, which started after a period of 15 mins, during which time the liver was allowed to 'settle down'. Each sample was 0.5 ml in volume and was replaced by an equal volume of perfusate. The perfusate volume was measured at the end of the experiment, so that any losses could be measured (such losses did not normally represent more than 5% of the perfusate volume at the end of the 130 min period).

Substrates or drugs were made up in 0.5 ml of Krebs bicarbonate medium and adjusted to a pH of 7.4 and brought up to a temperature of 37°C inside the cabinet before being added to the perfusate. All additions were placed into the reservoir below the animal platform, where a magnetic stirrer mixed them thoroughly with the perfusate. Substrates and drugs were added in the 0.5 ml volume in a concentration of 120-times the final required concentration, in order to compensate for the dilution in the 60 ml perfusate volume.

5). Treatment of Perfusate Samples.

The 0.5 ml perfusate samples were deproteinized with 1.5 ml of 7.5% trichloroacetic acid (TCA) and centrifuged at 2000 g, for 10 mins. The supernatant was analyzed for glucose, using the ferricyanide method - Technicon Autoanalyser Method N-16b.
Figure 5(i)
DIAGRAM OF APPARATUS FOR ISOLATED LIVER PERFUSION IN SITU
(Adapted from Hess et al. (2))

All apparatus in cabinet maintained at 37°C

Medium flows down bulb oxygenator

Filter (obtained from disposable blood transfusion set) to eliminate any clots in the medium

Medium flows up bulb oxygenator

Water-saturated 95% O₂/5% CO₂

Overflow to reservoir

Medium entering liver

Medium pumped from reservoir

Animal platform

Medium returning to reservoir after perfusing through liver

Drugs added to and samples taken from reservoir here

Reservoir

Magnetic stirrer
DIAGRAM OF OPERATIVE PREPARATION FOR LIVER PERFUSION IN SITU.

The method used was based on that of Seglen (19) and involved the sequential perfusion of the liver with, first, a Ca++-free buffer containing ethyleneglycol-bis-(β-amino-ethylether)N,N'-tetra-acetic acid (EGTA) and, second, a Ca++-buffer containing the enzymes collagenase and hyaluronidase.

1). Apparatus.

The apparatus for perfusing the liver to isolate the hepatocytes had to be adapted from the apparatus described for liver perfusions (see Fig 5 (i)), so that the liver was perfused in isolation from the body in order to collect the perfusion medium that leaked from the liver surface as the enzymes caused disruption of the liver structure. Two reservoirs also had to be incorporated into the apparatus for the two different perfusion media used to isolate the hepatocytes. The design of the apparatus can be seen in Fig. 5 (iii).

2). Perfusion and Incubation Media Employed.

(i). The first perfusion medium was a 0.5 mM EGTA, Ca++-free buffer made up as follows:

8.00 g NaCl ; 0.35 g KCl ; 0.16 g KH₂PO₄ ; 0.16 g MgSO₄·7H₂O ; 2.38 g HEPES buffer ; 0.19 g EGTA ; 51 mls 0.15 N NaOH and distilled water to make up to 1000 mls.

(ii). The second perfusion medium was a 10 mls stock enzyme solution + 10 mls 50 mM Ca++-buffer + 90 mls Ca++-free buffer, made up as follows:

a). Enzyme stock solution:

220 mgs collagenase Type II ; 50 mgs hyaluronidase Type V ; 30 mls Ca++-free buffer and filtered through a 0.22 μ Millipore filter.

b). 50 mM Ca++-buffer:

0.49 g NaCl ; 0.044 g KCl ; 0.013 g MgCl₂·6H₂O ; 0.735 g CaCl₂·2H₂O ; 0.238 g HEPES ; 3.6 mls 0.15 N NaOH ; distilled water to make up to 100 mls.

c). Ca++-free buffer:

8.18 g NaCl ; 0.35 g KCl ; 0.16 g KH₂PO₄ ; 0.16 g MgSO₄·7H₂O ; 2.38 g HEPES ; 45 mls 0.15 N NaOH ; distilled water to make up to 1000 mls.
operative procedure.

The rats used were 110-140 gm. Charles River CD male rats. They were anaesthetized with Nembutal (0.15 mls of 6% solution per 100 gm body weight) administered by the intraperitoneal route. The liver, portal vein and bile duct were exposed as described in the Methods for the in situ liver perfusion. The rats were then heparinized. Ligatures were placed around the gastrohepatic and gastroduodenal ligaments, two around the latter to include the pancreatico-duodenal vessels, and the ligaments were divided. In addition, other connections of the liver were divided; these preliminaries permitted the liver to be removed with the minimum of delay when cannulation was completed.

The bile duct was then cannulated and cut beyond the point of the cannulation. The portal vein was cannulated and three ligatures were tied as shown in Fig 5(ii). Immediately after this cannulation, the first perfusion was started through the portal vein. The portal vein was cut between the two ligatures holding the cannula in place and the third ligature. The inferior vena cava was cut between the heart and the diaphragm by cutting through the diaphragm to leave only a small collar of it attached to the vein. The liver could then be removed and placed in the perfusing apparatus. It was found unnecessary in perfusions (for the purpose of isolating liver cells) to cannulate the inferior vena cava.

4). Isolation of the Hepatocytes.

The first perfusion medium consisted of 150 mls of 0.5 mM EGTA, Ca\(^{++}\)-free buffer. The liver was perfused with this medium immediately after cannulation of the hepatic portal vein at 37ºC at the rate of 30 mls/min. The first 50 mls of medium were discarded via a 3-way tap [No. 1 on Fig. 5(iii)] to the drains, having been perfused through the liver. The perfusion was then switched to the recirculating system, so that the medium was returned to its reservoir prior to being pumped back to the liver via the oxygenator. After 10 mins of this initial perfusion, the four 3-way taps in the system were switched so that the medium in reservoir 2 was perfused through the liver. The first 10 mls of the second perfusion was
contaminate the enzyme-containing medium. Following this, the perfusion was switched to a recirculating system, which was continued until the appearance of the liver indicated that the structure had been disrupted (touching the liver surface with a spatula would cause the cells below to move, the whole liver consistency became soft and, by the end of the second perfusion, the medium was leaking from the surface of the liver lobes - this usually occurred within 10 mins).

The liver was then carefully lifted out of the perfusion apparatus and placed in a Petri dish containing Krebs-bicarbonate solution. The liver membrane was broken and the cells dispersed by gentle agitation with a glass rod. The resulting suspension was filtered through a 30 μm pore size filter to remove any debris and large clumps of cells. The filtered suspension was centrifuged at 50g, for 2 mins, and the supernatant poured away. The cell plug was resuspended in Krebs-bicarbonate medium and then recentrifuged at 50g, for 2 mins. The wet weight of the hepatocytes was measured after the final centrifugation and removal of the supernatant, and the cells were then resuspended at a concentration of 100-150mg/ml in Krebs-bicarbonate medium (this is equivalent to 8-10x10⁶ cells/ml - (41).

5). Viability of the Cell Preparation.

The viability of the hepatocyte preparation was measured as the % of cells which resisted trypan blue uptake (19, 21). A 0.5% solution of trypan blue in Krebs-bicarbonate medium was filtered through a 0.22 μm Millipore filter and one drop mixed with one drop of cell suspension. The proportion of stained to unstained cells was measured under a light microscope. Cell viability, as measured by this method, was normally about 90% in the preparations used in this study. This figure is similar to the findings of Seglen (19).

6). Incubations with the Cell Suspension.

All incubations were carried out in 25 ml conical beakers in a water bath at 37°C with the shaker set at 120 rpm. Each incubation beaker contained a total volume of 0.7 mls of which 0.5 mls was the cell
suspension. In the remaining 0.2 mls was a 3.5-times the desired final concentration of the additive (ie: substrates/drugs). All the additives were dissolved in the Krebs-bicarbonate medium, except glucagon which was made up in 0.01 M NaOH - in this latter case, the controls also had 0.2 mls of 0.01 M NaOH added instead of 0.2 mls of Krebs-bicarbonate medium.

Incubations were stopped by the addition of 0.7 mls of ice-cold 10% TCA solution and were immediately centrifuged at 2000 g, for 10 mins. Samples of the supernatant were used for glucose analysis by the Autoanalyzer and the results were expressed as μmoles glucose formed/gm. wet liver cell weight.
Figure 5(iii).

DIAGRAM OF APPARATUS FOR PERFUSION OF LIVER WITH MEDIA FOR ISOLATING HEPATOCYTES.

Medium pumped from appropriate reservoir to oxygenator via 3-way tap.

Water at 37°C into inner jacket of oxygenator.

Medium flows over inner jacket of oxygenator.

Overflow to appropriate reservoir controlled by 3-way tap.

Water saturated 95% O₂/5% CO₂.

(1) 3-way tap to liver.

Reservoir 1

Reservoir 2

Enclosed liver container.

The reservoirs and liver container were kept in a water bath at 37°C.
Results.

a). Gluconeogenesis by Isolated Perfused Rat Livers.

The experimental results described in this section illustrate the effects of insulin, phenformin and Wy 23675 on gluconeogenesis by perfused livers prepared from fasted rats.

Gluconeogenesis from lactate (10 mM) plus pyruvate (1 mM) and from alanine (10 mM) is illustrated in Figures 5(iv) and 5(v), respectively. The rate of formation of glucose after the addition of lactate plus pyruvate to the perfusate was approximately 62 μmoles/gm. wet liver weight/hr, while in the control perfusions with no exogenous lactate plus pyruvate it was approximately 17 μmoles/gm. wet liver weight/hr. The addition of alanine resulted in rates of glucose formation of approximately 24 μmoles/gm. wet liver weight/hr. - this was not markedly greater than the control levels.

Glucagon stimulated gluconeogenesis from both lactate plus pyruvate (Figure 5(vi)) and alanine (Figure 5(vii)); in both cases the magnitude of stimulation was approximately 3-fold. Insulin had no effect on the rate of gluconeogenesis from lactate plus pyruvate in these experiments (Figure 5(viii)). However, both phenformin (Figure 5(ix)) and Wy 23675 (Figure 5(x)) inhibited gluconeogenesis from lactate plus pyruvate to approximately one-third of the control values.

b). Glycogenolysis by Isolated Hepatocytes Prepared from Normal, Fed Rats.

The results described in this section illustrate the effects of glucagon, insulin, tolbutamide, phenformin and Wy 23675 on glucose production by isolated hepatocytes prepared from normal, fed rats and are summarized in Table 5(i). No exogenous gluconeogenic substrate was added to any of these incubations.

The basal rate of glucose production from these preparations was approximately 63 μmoles/gm. wet liver cell weight/hr (Figure 5(xi)). Glucagon, at all concentrations used (50nM-5μM), significantly stimulated the production of glucose; the highest concentration used (5μM)
stimulated glucose production by approximately 2-fold. Insulin, in concentrations from 4 μUnits/ml to 400 μUnits/ml, had no effect on basal glucose production, but did decrease the stimulation of glucose production caused by 500 nM glucagon, although not back to control levels. Tolbutamide, in concentrations from 0.2mM to 20mM, had no effect on either basal or glucagon-stimulated glucose production. Phenformin and Wy 23675, in the same concentrations as tolbutamide, also had no effect on either basal or glucagon-stimulated glucose production.

c. Gluconeogenesis by Isolated Hepatocytes Prepared from Normal, Fasted Rats.

The results described in this section illustrate the effects of glucagon, insulin, tolbutamide, phenformin and Wy 23675 on gluconeogenesis by isolated hepatocytes prepared from normal, fasted rats and are summarized in Table 5(ii).

Basal rates of gluconeogenesis from lactate plus pyruvate, alanine, dihydroxyacetone and xylitol were established (65, 31, 93, 141 μmoles glucose formed/gm. wet liver cell weight/hr, respectively). These rates were comparable to those obtained in experiments using perfused livers (ie: gluconeogenesis from lactate plus pyruvate in the perfused liver was 62 μmoles glucose/gm. wet liver cell weight/hr compared with 65 μmoles glucose/gm. wet liver cell weight/hr in isolated liver cells; similarly, gluconeogenesis from alanine in the perfused liver was 24 μmoles glucose/gm. wet liver weight/hr compared with 31 μmoles/gm. wet liver cell weight/hr in isolated liver cells).

Glucagon (5μM) stimulated gluconeogenesis from lactate plus pyruvate and from alanine by approximately 2.5-fold, while it stimulated by approximately 1.7-fold from dihydroxyacetone and had no effect on gluconeogenesis from xylitol. Insulin had no effect on the rate of gluconeogenesis from lactate plus pyruvate — this was similar to the finding in perfused livers — but it did inhibit a submaximal glucagon-stimulation of gluconeogenesis from lactate plus pyruvate in a dose-related manner.
Tolbutamide had no effects on either the basal rate of gluconeogenesis from lactate plus pyruvate or on submaximal glucagon-stimulated gluconeogenesis from lactate plus pyruvate. In contrast, phenformin inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, while having no effect on gluconeogenesis from xylitol. Similarly, Wy 23675 inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but had no effect on gluconeogenesis from xylitol. However, it should be noted that the concentrations of phenformin required to inhibit rat liver gluconeogenesis (2mM) are approximately 100-fold higher than those normally achieved in the plasma of patients on therapeutic doses of phenformin (52-54). When gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone was submaximally-stimulated by glucagon, phenformin had inhibitory effects in concentrations as low as 2μM. Wy 23675 also inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone in lower doses when it was submaximally-stimulated by glucagon than under basal conditions.


Comparisons were made of the rates of gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone and from xylitol in hepatocytes prepared from normal, fasted and alloxan-diabetic, fasted rats (Table 5(iii)). In all instances, the rate of gluconeogenesis was greater in hepatocytes prepared from alloxan-diabetic rats than from normal rats. The increase was approximately 1.5-times with regard to gluconeogenesis from both lactate plus pyruvate and from dihydroxyacetone and approximately 1.3-times with regard to gluconeogenesis from xylitol.

e). Gluconeogenesis by Isolated Hepatocytes Prepared from Fasted, Alloxan-Diabetic Rats.

The results described in this section illustrate the effects of glucagon, insulin, tolbutamide, phenformin and Wy 23675 on gluconeogenesis by isolated hepatocytes prepared from fasted, alloxan-diabetic rats.
Glucagon was able to further stimulate gluconeogenesis from lactate plus pyruvate (Table 5(iv)). Insulin appeared to inhibit gluconeogenesis from lactate plus pyruvate in a dose-related manner (Table 5(iv)), but these results were not statistically significant (Students t-Test). However, when alloxan-diabetic rats were treated with insulin in vivo for 3 days prior to the preparation of the isolated hepatocytes, the rate of gluconeogenesis from lactate plus pyruvate was inhibited by approximately 50% as compared with the controls (Table 5(v)). In similar experiments, insulin treatment in vivo was shown to inhibit gluconeogenesis from dihydroxyacetone by approximately 50% and from xylitol by approximately 40% (Table 5(v)).

Tolbutamide had no effect on gluconeogenesis from lactate plus pyruvate either in vitro (Table 5(iv)) or when administered in vivo for three days prior to the preparation of isolated hepatocytes (Table 5(v)). In contrast, phenformin inhibited gluconeogenesis from both lactate plus pyruvate and from dihydroxyacetone, but had no effect on gluconeogenesis from xylitol in vitro (Table 5(iv)). The concentration of phenformin required for an inhibitory effect on gluconeogenesis in hepatocytes prepared from alloxan-diabetic rats was 100-fold less than that required for inhibitory effects in hepatocytes prepared from normal rats (eg: Compare Table 5(ii) with Table 5(iv)). Wy 23675, like phenformin, inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but had no effect on gluconeogenesis from xylitol (Table 5(iv)).
THE ACTIONS OF SELECTED AGENTS ON GLUCOSE PRODUCTION BY THE RAT LIVER.

**Figure No.**

5(i) Diagram of Apparatus for Isolated Liver Perfusion 'in Situ'.

5(ii) Diagram of Operative Preparation for Liver Perfusion 'in Situ'.

5(iii) Diagram of Apparatus for Perfusion of Liver with Media for Isolating Liver Cells.

5(iv) Effect of Lactate plus Pyruvate on Gluconeogenesis by Perfused Livers from Fasted Rats.

5(v) Effect of Alanine on Gluconeogenesis by Perfused Livers from Fasted Rats.

5(vi) Effect of Glucagon on Lactate plus Pyruvate-Stimulated Gluconeogenesis by Perfused Livers from Fasted Rats.

5(vii) Effect of Glucagon on Alanine-Stimulated Gluconeogenesis by Perfused Livers from Fasted Rats.

5(viii) Effect of Insulin on Lactate plus Pyruvate-Stimulated Gluconeogenesis by Perfused Livers from Fasted Rats.

5(ix) Effect of Phenformin on Lactate plus Pyruvate-Stimulated Gluconeogenesis by Perfused Livers from Fasted Rats.

5(x) Effect of Wy 23675 on Lactate plus Pyruvate-Stimulated Gluconeogenesis by Perfused Livers from Fasted Rats.

5(xi) Glucose Production by Isolated Hepatocytes Prepared from a Normal, Fed Rat.

**Table No.**

5(i) Glycogenolysis by Isolated Hepatocytes Prepared from Normal, Fed Rats.

5(ii) Gluconeogenesis by Isolated Hepatocytes Prepared from Normal, Fasted Rats.

5(iii) Comparison of Rates of Gluconeogenesis by Isolated Hepatocytes Prepared from Fasted, Normal and Alloxan-Diabetic Rats.
Gluconeogenesis by Isolated Hepatocytes Prepared from Fasted, Alloxan-Diabetic Rats.

Effect of In Vivo Administration of Drugs on Gluconeogenesis by Isolated Hepatocytes Prepared from Fasted, Alloxan-Diabetic Rats.
Livers from 18 hr fasted rats were perfused in situ for 55 mins with a recirculating medium, containing no substrate, as described in Methods. Perfusions were continued for a further 70 mins in the absence (controls) or presence of 10 mM lactate plus 1 mM pyruvate. 0.5 ml samples of the perfusion medium were taken from the reservoir for glucose analysis at 10 minute intervals, beginning 15 mins after the start of the perfusion. Each 0.5 ml sample was replaced with 0.5 ml of the red blood cell/Krebs medium. The results are expressed in umoles of glucose formed/gm wet liver weight. 3 control perfusions (■) were compared with 4 to which lactate/pyruvate had been added (▲). Each point on the figure represents the mean ± the standard error of the mean for these perfusions.

Glucose Formed
(µmoles/gm. wet liver weight)

Krebs bicarbonate added to control group; lactate plus pyruvate added to test group.
EFFECT OF ALANINE 10mM ON GLUCONEOGENESIS BY PERFUSED LIVERS FROM FASTED RATS.

Livers from 18 hr fasted rats were perfused in situ for 55 mins with a recirculating medium containing no substrate, as described in Methods. Per fusions were continued for a further 70 mins in the absence (controls) or presence of 10 mM alanine. Other details are as described in Figure 5(iv). 3 control perfusions (■) were compared with 4 to which alanine had been added (▲). Each point on the figure represents the mean ± the standard error of the mean for these perfusions.

![Graph showing glucose formed over time with and without alanine addition.](image-url)
Livers from 48 hr fasted rats were perfused in situ (as described in Methods) for 55 min with a recirculating medium containing 10 mM lactate plus 1 mM pyruvate before the addition of glucagon (in a final concentration of 50 nM in the perfusion medium). Other details are as described in Figure 5(iv). This figure shows the mean ± the standard error of the mean of 4 such perfusions.

**Figure 5(vi).**

Glucose Formed
(μmoles/gm. wet liver weight)
Livers from 18 hr fasted rats were perfused in situ (as described in Methods) for 55 mins with a recirculating medium containing 10 mM alanine before the addition of glucagon (in a final concentration of 50 nM in the perfusion medium). Other details are as described in Figure 5(iv). This figure shows the mean ± the standard error of the mean of 4 such perfusions.

Glucose Formed
(μmoles/gm. wet liver weight)

10mM alanine added at zero time

Glucagon 50nM added

Extrapolation of gluconeogenesis from before glucagon addition

Time (mins)
Livers from 18 hr fasted rats were perfused in situ (as described in Methods) for 55 mins with a recirculating perfusion medium containing 10 mM lactate plus 1 mM pyruvate before the addition of insulin (final concentration of 40 μUnits/ml in the perfusion medium) Other details are as described in Figure 5(iv). This figure shows the mean ± the standard error of the mean of 4 such perfusions.

Glucose Formed
(μmoles/gm. wet liver weight)

- 10 mM lactate + 1 mM pyruvate added at zero time
- Insulin 40μU/ml added
EFFECT OF PHENFORMIN ON LACTATE + PYRUVATE-STIMULATED GLUCONEOGENESIS
BY PERFUSED LIVERS FROM FASTED RATS.

Livers from 18 hr fasted rats were perfused in situ (as described in Methods) for 55 mins with a recirculating medium containing 10 mM lactate plus 1 mM pyruvate before the addition of phenformin (final concentration of 2 mM in the perfusion medium). Other details are as described in Figure 5(iv). This figure shows the mean ± the standard error of the mean of 4 such perfusions.

Glucose Formed
(µmoles/gm, wet liver weight)
140 120 100 80 60 40 20

10 mM lactate + 1 mM pyruvate
added at zero time

Extrapolation of gluconeogenesis
from before phenformin
addition

Phenformin 2 mM
added

Time (mins)
0 15 25 35 45 55 65 75 85 95 105 115 125
Livers from 18 hr fasted rats were perfused in situ (as described in Methods) for 55 mins with a recirculating medium containing 10 mM lactate plus 1 mM pyruvate before the addition of Wy 23675 (final concentration of 2 mM in the perfusion medium). Other details were as described in Figure 5(iv). This figure shows the mean ± the standard error of the mean of 4 such perfusions.
Isolated hepatocytes were prepared from fed rats and incubated as described in Methods. Incubations were stopped at the time points shown and the glucose content of the medium was measured and expressed in μmoles/gm wet liver cell weight. No additions were made to these incubations. Each time point represents the mean ± the standard error of 6 observations.
Glucose Production (μmoles/gm wet liver cell weight) for the Following Incubation Times (mins):

<table>
<thead>
<tr>
<th></th>
<th>Glucose Production (μmoles/gm wet liver cell weight)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>30 (±5)</td>
</tr>
<tr>
<td>Glucagon 50nM</td>
<td>39 (±7)</td>
</tr>
<tr>
<td>Glucagon 500nM</td>
<td>56 (±7)*</td>
</tr>
<tr>
<td>Glucagon 5μM</td>
<td>64 (±4)***</td>
</tr>
<tr>
<td>Control</td>
<td>39 (±8)</td>
</tr>
<tr>
<td>Insulin 4μU/ml</td>
<td>35 (±10)</td>
</tr>
<tr>
<td>Insulin 40μU/ml</td>
<td>33 (±7)</td>
</tr>
<tr>
<td>Insulin 400μU/ml</td>
<td>31 (±6)</td>
</tr>
<tr>
<td>Control</td>
<td>22 (±5)</td>
</tr>
<tr>
<td>Glucagon 500nM</td>
<td>46 (±4)**</td>
</tr>
<tr>
<td>Glucagon 500nM + Insulin 40μU/ml</td>
<td>30 (±7)</td>
</tr>
<tr>
<td>Control</td>
<td>27 (±6)</td>
</tr>
<tr>
<td>Tolbutamide 0.2mM</td>
<td>33 (±4)</td>
</tr>
<tr>
<td>Tolbutamide 2mM</td>
<td>29 (±6)</td>
</tr>
<tr>
<td>Tolbutamide 20mM</td>
<td>22 (±8)</td>
</tr>
<tr>
<td>Control</td>
<td>38 (±4)</td>
</tr>
<tr>
<td>Glucagon 500nM</td>
<td>55 (±8)</td>
</tr>
<tr>
<td>Glucagon 500nM + Tolbutamide 20mM</td>
<td>61 (±6)**</td>
</tr>
</tbody>
</table>

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

* p<0.05 ;  ** p<0.01 ;  *** p<0.001
**GLYCOGENOLYSIS BY ISOLATED HEPATOCYTES PREPARED FROM NORMAL, FED RATS.**

<table>
<thead>
<tr>
<th></th>
<th>Glucose Production (μmoles/gm, wet liver cell weight) for the Following Incubation Times (mins):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>19 (±6)</td>
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<tr>
<td>Phenformin 0.2mM</td>
<td>23 (±3)</td>
</tr>
<tr>
<td>Phenformin 2mM</td>
<td>17 (±4)</td>
</tr>
<tr>
<td>Phenformin 20mM</td>
<td>21 (±3)</td>
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<tr>
<td>Control</td>
<td>27 (±6)</td>
</tr>
<tr>
<td>Glucagon 500nM</td>
<td>72 (±11)**</td>
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<td>Glucagon 500nM +</td>
<td>61 (±8)**</td>
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<td>63 (±5)**</td>
</tr>
<tr>
<td>Glucagon 500nM + Wy 23675 20mM</td>
<td>64 (±6)**</td>
</tr>
</tbody>
</table>

Statistically significant changes from the appropriate controls were determined by Student's t-Test:

- * p<0.05
- ** p<0.01
- *** p<0.001
Isolated hepatocytes prepared from 18-hr. fasted rats and incubated as described in Methods were used. Incubations were stopped at the time points shown and the glucose content of the medium was measured and expressed in μmoles/gm. wet liver cell weight. Each value represents the mean ± the standard error of 6 observations. Statistically significant changes from the appropriate controls were determined by Student’s t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001

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<th>Glucose Production (μmoles/gm. wet liver cell weight) for the Following Incubation Times (mins):</th>
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<tbody>
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<td>20</td>
</tr>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>Lactate 10 mM +</td>
<td>14 (±6)</td>
</tr>
<tr>
<td>Pyruvate 1 mM</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6 (±3)</td>
</tr>
<tr>
<td>Alanine 10 mM</td>
<td>9 (±3)</td>
</tr>
<tr>
<td>Control</td>
<td>5 (±2)</td>
</tr>
<tr>
<td>Dihydroxyacetone 10 mM</td>
<td>28 (±4)**</td>
</tr>
<tr>
<td>Control</td>
<td>6 (±2)</td>
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<tr>
<td>Xylitol 10 mM</td>
<td>34 (±6)**</td>
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<tr>
<td>Glucagon 5μM</td>
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<td>Lactate 10 mM +</td>
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<td>Pyruvate 1 mM (L/P)</td>
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<td>L/P + Glucagon 50nM</td>
<td>14 (±2)</td>
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<tr>
<td>L/P + Glucagon 5μM</td>
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<tr>
<td>Alanine 10 mM</td>
<td>8 (±2)</td>
</tr>
<tr>
<td>Alanine 10 mM +</td>
<td>20 (±4)*</td>
</tr>
<tr>
<td>Glucagon 5μM</td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone 10 mM (D)</td>
<td>29 (±3)</td>
</tr>
<tr>
<td>D + Glucagon 50nM</td>
<td>42 (±3)*</td>
</tr>
<tr>
<td>D + Glucagon 5μM</td>
<td>46 (±5)*</td>
</tr>
<tr>
<td>Xylitol 10 mM (X)</td>
<td>40 (±3)</td>
</tr>
<tr>
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<td>X + Glucagon 5μM</td>
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</table>
GLUCONEOGENESIS BY ISOLATED HEPATOCYTES PREPARED FROM NORMAL, FASTED RATS.

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<tr>
<th>Glucose Production (μmoles/gm. wet liver cell weight)</th>
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<th>60</th>
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<tbody>
<tr>
<td>Lactate 10 mM + Pyruvate 1 mM (L/P)</td>
<td>20 (±4)</td>
<td>33 (±4)</td>
<td>59 (±5)</td>
</tr>
<tr>
<td>L/P + Insulin 40 μUnits/ml</td>
<td>18 (±2)</td>
<td>36 (±3)</td>
<td>57 (±7)</td>
</tr>
<tr>
<td>L/P + Insulin 400 μUnits/ml</td>
<td>22 (±3)</td>
<td>28 (±5)</td>
<td>59 (±4)</td>
</tr>
<tr>
<td>Lactate 10 mM + Pyruvate 1 mM + Glucagon 50nM (L/P/G)</td>
<td>39 (±6)</td>
<td>75 (±9)</td>
<td>141 (±16)</td>
</tr>
<tr>
<td>L/P/G + Insulin 4 μUnits/ml</td>
<td>41 (±4)</td>
<td>68 (±7)</td>
<td>130 (±11)</td>
</tr>
<tr>
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<td>18 (±3)</td>
<td>44 (±6)</td>
<td>71 (±5)</td>
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<tr>
<td>L/P + Tolbutamide 0.2 mM</td>
<td>20 (±4)</td>
<td>51 (±5)</td>
<td>65 (±5)</td>
</tr>
<tr>
<td>L/P + Tolbutamide 2 mM</td>
<td>17 (±2)</td>
<td>47 (±5)</td>
<td>69 (±4)</td>
</tr>
<tr>
<td>L/P + Tolbutamide 20 mM</td>
<td>22 (±3)</td>
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<td>62 (±7)</td>
</tr>
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<td>51 (±8)</td>
<td>111 (±10)</td>
</tr>
<tr>
<td>L/P/G + Tolbutamide 0.2 mM</td>
<td>16 (±4)</td>
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</tr>
<tr>
<td>L/P/G + Tolbutamide 2 mM</td>
<td>19 (±6)</td>
<td>45 (±4)</td>
<td>122 (±15)</td>
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<td>103 (±12)</td>
</tr>
<tr>
<td>Lactate 10 mM + Pyruvate 1 mM (L/P)</td>
<td>18 (±3)</td>
<td>38 (±5)</td>
<td>60 (±5)</td>
</tr>
<tr>
<td>L/P + Phenformin 0.2 mM</td>
<td>17 (±2)</td>
<td>41 (±4)</td>
<td>56 (±6)</td>
</tr>
<tr>
<td>L/P + Phenformin 2 mM</td>
<td>14 (±2)</td>
<td>30 (±3)</td>
<td>44 (±5)*</td>
</tr>
<tr>
<td>L/P + Phenformin 20 mM</td>
<td>8 (±2)*</td>
<td>17 (±4)**</td>
<td>23 (±4)***</td>
</tr>
<tr>
<td>Dihydroxyacetone 10 mM (D)</td>
<td>27 (±3)</td>
<td>67 (±4)</td>
<td>103 (±9)</td>
</tr>
<tr>
<td>D + Phenformin 0.2 mM</td>
<td>32 (±4)</td>
<td>72 (±7)</td>
<td>111 (±10)</td>
</tr>
<tr>
<td>D + Phenformin 2 mM</td>
<td>21 (±3)</td>
<td>47 (±5)*</td>
<td>75 (±9)</td>
</tr>
<tr>
<td>D + Phenformin 20 mM</td>
<td>17 (±4)</td>
<td>39 (±7)**</td>
<td>61 (±11)*</td>
</tr>
</tbody>
</table>

Statistically significant changes from the appropriate controls were determined by Student's t-Test:—

* p<0.05 ; ** p<0.01 ; *** p<0.001

200
GLUCONEOGENESIS BY ISOLATED HEPATOCYTES PREPARED FROM NORMAL, FASTED RATS.

<table>
<thead>
<tr>
<th>Glucose Production (pmoles/gm, wet liver cell weight) for the Following Incubation Time (mins):-</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylitol 10 mM (X)</td>
<td>47 (±5)</td>
<td>103 (±11)</td>
<td>159 (±12)</td>
</tr>
<tr>
<td>X + Phenformin 0.2 mM</td>
<td>43 (±6)</td>
<td>96 (±11)</td>
<td>147 (±17)</td>
</tr>
<tr>
<td>X + Phenformin 2 mM</td>
<td>57 (±6)</td>
<td>112 (±15)</td>
<td>161 (±19)</td>
</tr>
<tr>
<td>X + Phenformin 20 mM</td>
<td>38 (±7)</td>
<td>99 (±12)</td>
<td>153 (±15)</td>
</tr>
<tr>
<td>Lactate 10 mM + Pyruvate 1 mM (I/P)</td>
<td>23 (±2)</td>
<td>44 (±5)</td>
<td>73 (±8)</td>
</tr>
<tr>
<td>I/P + Wy 23675 0.2 mM</td>
<td>25 (±2)</td>
<td>41 (±4)</td>
<td>64 (±7)</td>
</tr>
<tr>
<td>I/P + Wy 23675 2 mM</td>
<td>19 (±2)</td>
<td>32 (±5)</td>
<td>57 (±6)</td>
</tr>
<tr>
<td>I/P + Wy 23675 20 mM</td>
<td>10 (±2)***</td>
<td>24 (±2)***</td>
<td>33 (±4)***</td>
</tr>
<tr>
<td>Dihydroxyacetone 10 mM (D)</td>
<td>30 (±2)</td>
<td>71 (±7)</td>
<td>112 (±11)</td>
</tr>
<tr>
<td>D + Wy 23675 0.2 mM</td>
<td>28 (±4)</td>
<td>72 (±4)</td>
<td>104 (±29)</td>
</tr>
<tr>
<td>D + Wy 23675 2 mM</td>
<td>21 (±3)**</td>
<td>50 (±6)***</td>
<td>76 (±8)***</td>
</tr>
<tr>
<td>D + Wy 23675 20 mM</td>
<td>17 (±3)**</td>
<td>34 (±5)***</td>
<td>57 (±6)***</td>
</tr>
<tr>
<td>Xylitol 10 mM (X)</td>
<td>41 (±6)</td>
<td>108 (±10)</td>
<td>167 (±18)</td>
</tr>
<tr>
<td>X + Wy 23675 0.2 mM</td>
<td>39 (±4)</td>
<td>107 (±9)</td>
<td>154 (±12)</td>
</tr>
<tr>
<td>X + Wy 23675 2 mM</td>
<td>44 (±5)</td>
<td>98 (±10)</td>
<td>147 (±13)</td>
</tr>
<tr>
<td>X + Wy 23675 20 mM</td>
<td>33 (±4)</td>
<td>96 (±6)</td>
<td>144 (±11)</td>
</tr>
<tr>
<td>Lactate 10 mM + Pyruvate 1 mM + Glucagon 50nM (I/P/G)</td>
<td>31 (±3)</td>
<td>70 (±5)</td>
<td>103 (±9)</td>
</tr>
<tr>
<td>I/P/G + Phenformin 2 μM</td>
<td>17 (±3)**</td>
<td>37 (±5)***</td>
<td>50 (±6)***</td>
</tr>
<tr>
<td>I/P/G + Phenformin 20 μM</td>
<td>8 (±2)***</td>
<td>22 (±3)***</td>
<td>30 (±5)***</td>
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<tr>
<td>I/P/G + Phenformin 0.2 mM</td>
<td>10 (±2)***</td>
<td>25 (±2)***</td>
<td>33 (±3)***</td>
</tr>
<tr>
<td>I/P/G + Phenformin 2 mM</td>
<td>8 (±1)***</td>
<td>18 (±3)***</td>
<td>24 (±2)***</td>
</tr>
<tr>
<td>Dihydroxyacetone 10 mM + Glucagon 50nM (D/G)</td>
<td>59 (±4)</td>
<td>111 (±12)</td>
<td>163 (±15)</td>
</tr>
<tr>
<td>D/G + Phenformin 2 μM</td>
<td>29 (±4)***</td>
<td>62 (±6)***</td>
<td>86 (±9)***</td>
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<td>D/G + Phenformin 2 mM</td>
<td>20 (±3)***</td>
<td>33 (±3)***</td>
<td>52 (±7)***</td>
</tr>
</tbody>
</table>

Statistical significance was determined by Student's T-test:-
* P<0.05 ; ** P<0.01 ; *** P<0.001
**GLUCONEOGENESIS BY ISOLATED HEPATOCYTES PREPARED FROM NORMAL, FASTED RATS.**

<table>
<thead>
<tr>
<th>Glucose Production (µmoles/gm. wet liver cell weight) for the Following Incubation Time (mins):-</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate 10 mM + Pyruvate 1mM + Glucagon 50nM (L/P/G)</td>
<td>36 (±3)</td>
<td>82 (±9)</td>
<td>116 (±12)</td>
</tr>
<tr>
<td>L/P/G + Wy 23675 2 µM</td>
<td>15 (±2)***</td>
<td>39 (±4)***</td>
<td>51 (±6)***</td>
</tr>
<tr>
<td>L/P/G + Wy 23675 20 µM</td>
<td>9 (±2)***</td>
<td>22 (±2)***</td>
<td>29 (±4)***</td>
</tr>
<tr>
<td>L/P/G + Wy 23675 0.2 mM</td>
<td>8 (±1)***</td>
<td>19 (±3)***</td>
<td>23 (±2)***</td>
</tr>
<tr>
<td>L/P/G + Wy 23675 2 mM</td>
<td>6 (±1)***</td>
<td>16 (±3)***</td>
<td>14 (±2)***</td>
</tr>
<tr>
<td>Dihydroxyacetone 10 mM + Glucagon 50nM (D/G)</td>
<td>44 (±3)</td>
<td>100 (±11)</td>
<td>147 (±15)</td>
</tr>
<tr>
<td>D/G + Wy 23675 2 µM</td>
<td>23 (±3)***</td>
<td>60 (±7)****</td>
<td>82 (±9)***</td>
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<tr>
<td>D/G + Wy 23675 20 µM</td>
<td>16 (±2)***</td>
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<td>D/G + Wy 23675 0.2 mM</td>
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</table>

Statistical significance was determined by Student’s T-test:-

* p<0.05; ** p<0.01; *** p<0.001
**Table 5(iii).**

Comparison of rates of gluconeogenesis by isolated hepatocytes prepared from fasted, normal and alloxan-diabetic rats.

Test rats were treated with alloxan (0.7 mmol/kg i.p.) and those with blood glucose levels in excess of 16.5 mmol/l, two days later were used (control rats received no treatment). Isolated hepatocytes were prepared from 18-hr. fasted rats and incubated as described in Methods. Each value represents the mean (± the standard error) of 6 observations.

Statistical significance was determined by Student's t-Test:­

* p<0.05 ; ** p<0.01 ; *** p<0.001

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<tr>
<td><strong>Lactate 10 mM + Pyruvate 1 mM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Rats</td>
<td>14 (±6)</td>
<td>26 (±5)</td>
<td>65 (±7)</td>
</tr>
<tr>
<td>Alloxan-diabetic Rats</td>
<td>27 (±4)</td>
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<td><strong>Dihydroxyacetone 10 mM</strong></td>
<td></td>
<td></td>
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<td>Normal Rats</td>
<td>28 (±4)</td>
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<td>Alloxan-diabetic Rats</td>
<td>39 (±5)</td>
<td>84 (±8)</td>
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</tr>
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<td>34 (±6)</td>
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</tr>
<tr>
<td>Alloxan-diabetic Rats</td>
<td>44 (±5)</td>
<td>124 (±10)</td>
<td>186 (±14)*</td>
</tr>
</tbody>
</table>
GLUCONEOGENESIS BY ISOLATED HEPATOCYTES PREPARED FROM FASTED, ALLOXAN-DIABETIC RATS.

Rats were treated with alloxan (0.7 mmol/kg i.p.) and those with blood glucose levels in excess of 16.5 mmol/l two days later. Isolated hepatocytes were prepared from 18 hr. fasted rats and incubated as described in Methods.

Each value represents the mean ± the standard error of 6 observations.

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<tr>
<th>Glucose Production (µmoles/gm, wet liver cell weight)</th>
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<tr>
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</tr>
<tr>
<td>L/P + Insulin 400 μU/ml</td>
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</tr>
<tr>
<td>Lactate 10 mM + Pyruvate 1 mM (L/P)</td>
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</tr>
<tr>
<td>L/P + Tolbutamide 0.2 mM</td>
<td>41 (±5)</td>
</tr>
<tr>
<td>L/P + Tolbutamide 2 mM</td>
<td>38 (±6)</td>
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<tr>
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</tr>
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<tr>
<td>L/P + Phenformin 0.2 mM</td>
<td>14 (±3)**</td>
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<td>Dihydroxyacetone 10 mM (D)</td>
<td>51 (±7)</td>
</tr>
<tr>
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<tr>
<td>D + Phenformin 20 μM</td>
<td>27 (±5)*</td>
</tr>
<tr>
<td>D + Phenformin 0.2 mM</td>
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</tr>
<tr>
<td>Xylitol 10 mM (X)</td>
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</tr>
<tr>
<td>X + Phenformin 2 μM</td>
<td>59 (±7)</td>
</tr>
<tr>
<td>X + Phenformin 20 μM</td>
<td>54 (±5)</td>
</tr>
<tr>
<td>X + Phenformin 0.2 mM</td>
<td>49 (±6)</td>
</tr>
</tbody>
</table>

Statistical significance was determined by Student’s t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
GLUCONEOGENESIS BY ISOLATED HEPATOCYTES PREPARED FROM FASTED, ALLOXAN-
DIABETIC RATS.

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<tr>
<td>L/P + Wy 23675 2 µM</td>
<td>15 (±3)*</td>
<td>31 (±4)**</td>
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<td>L/P + Wy 23675 20 µM</td>
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<td>155 (±17)</td>
</tr>
<tr>
<td>D + Wy 23675 2 µM</td>
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<td>80 (±9)</td>
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<td>191 (±20)</td>
</tr>
<tr>
<td>X + Wy 23675 2 µM</td>
<td>65 (±6)</td>
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<td>202 (±21)</td>
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<tr>
<td>X + Wy 23675 20 µM</td>
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<td>126 (±13)</td>
<td>186 (±19)</td>
</tr>
<tr>
<td>X + Wy 23675 0.2 mM</td>
<td>52 (±5)</td>
<td>109 (±11)</td>
<td>155 (±17)</td>
</tr>
</tbody>
</table>

Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
EFFECT OF IN VIVO ADMINISTRATION OF DRUGS ON GLUCONEOGENESIS BY ISOLATED HEPATOCYTES PREPARED FROM FASTED, ALLOXAN-DIABETIC RATS.

Rats were treated with alloxan (0.7 mmol/kg i.p.) and those with blood glucose levels in excess of 16.5 mmol/l. two days later were used. Control rats received no further treatment. Test rats received either 4 units of insulin per day by intramuscular injection or 0.2 mmol/kg p.o. of tolbutamide twice a day for three days prior to an experiment. Blood glucose levels were measured immediately prior to the preparation of the isolated hepatocytes and for the insulin-treated rats were 3.86-6.60 mmol/l. and for the tolbutamide-treated rats were 11.01-13.76 mmol/l. while controls were 18.75-25.62 mmol/l. Isolated hepatocytes were prepared from 18-hr. fasted rats and incubated as described in Methods. Each value represents the mean (± the standard error) of 6 observations.

<table>
<thead>
<tr>
<th>Glucose Production (µmoles/gm. wet liver cell weight) for the Following Incubation Times (mins):-</th>
<th>20</th>
<th>40</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactate 10 mM + Pyruvate 1 mM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>30 (±4)</td>
<td>83 (±10)</td>
<td>108 (±11)</td>
</tr>
<tr>
<td>Insulin-Treated (in vivo)</td>
<td>11 (±5)*</td>
<td>35 (±8)**</td>
<td>52 (±12)**</td>
</tr>
<tr>
<td><strong>Dihydroxyacetone 10 mM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50 (±8)</td>
<td>131 (±16)</td>
<td>172 (±19)</td>
</tr>
<tr>
<td>Insulin-Treated (in vivo)</td>
<td>26 (±6)*</td>
<td>63 (±11)**</td>
<td>86 (±21)*</td>
</tr>
<tr>
<td><strong>Xylitol 10 mM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55 (±7)</td>
<td>122 (±16)</td>
<td>184 (±20)</td>
</tr>
<tr>
<td>Insulin-Treated (in vivo)</td>
<td>32 (±9)</td>
<td>69 (±18)</td>
<td>109 (±15)*</td>
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<tr>
<td><strong>Lactate 10 mM + Pyruvate 1 mM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28 (±4)</td>
<td>63 (±7)</td>
<td>99 (±10)</td>
</tr>
<tr>
<td>Tolbutamide-Treated (in vivo)</td>
<td>25 (±5)</td>
<td>52 (±6)</td>
<td>78 (±9)</td>
</tr>
</tbody>
</table>

Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
Insulin, tolbutamide, phenformin and Wy 23675 have all been shown to possess hypoglycaemic properties. The effects of these agents on glucose utilization have been investigated and the results described in Chapters 2, 3 and 4. However, these agents have also been shown to lower blood glucose levels in 24-hr. fasted normal rats (see Table 1, Chapter 1) - a situation where gluconeogenesis is enhanced. The work in this chapter has investigated the effects of these agents on glucose production by the rat liver.

Comparison of Perfused Livers with Isolated Hepatocytes.

Most of the work reported in this chapter is with isolated rat hepatocytes. However, in order to justify their use, some of the results obtained with isolated hepatocytes have been compared with results from similar experiments with perfused livers (Table 5(vi)). Rates of gluconeogenesis from lactate plus pyruvate and from alanine were similar with either perfused livers or with isolated hepatocytes from starved rats. The rates of gluconeogenesis from these substrates compare closely with those reported by other workers for both perfused livers (2, 23, 24) and for isolated hepatocytes (21, 46).

Insulin had no effect on gluconeogenesis from lactate plus pyruvate with either the perfused liver or isolated hepatocytes. There is some controversy as to whether insulin has a direct inhibitory effect on basal gluconeogenesis or glycogenolysis in the liver (43, 45) or not (36, 37), or whether it acts in vivo by inhibiting the actions of glucagon and catecholamines. There seems to be general agreement that insulin acts in the isolated perfused liver in vitro to inhibit the glucagon- and adrenaline-stimulation of gluconeogenesis (33, 34, 38).

Isolated hepatocytes appeared to be less sensitive to the effects of glucagon, phenformin and Wy 23675 on gluconeogenesis than did perfused livers (Table 5(vi)). The percentage stimulation of gluconeogenesis by glucagon is similar to that reported by other workers for both the perfused liver (23, 35) and the isolated hepatocyte (21, 47-50).
<table>
<thead>
<tr>
<th>Substrate and Drugs Used</th>
<th>Glucose Production (µmoles/gm, wet liver weight/hr) by:</th>
<th>Perfused Rat Livers</th>
<th></th>
<th></th>
<th></th>
<th>Isolated Rat Hepatocytes</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose Production</td>
<td>% Change from Control</td>
<td>Figure No.</td>
<td>Glucose Production</td>
<td>% Change from Control</td>
<td>No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17 (±2)</td>
<td>5(iv)</td>
<td></td>
<td>15 (±2)</td>
<td>65 (±7)</td>
<td>+333%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate 10mM + Pyruvate 1mM (I/P)</td>
<td>63 (±4)</td>
<td>+271%</td>
<td></td>
<td>65 (±7)</td>
<td>+333%</td>
<td>5(ii)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17 (±2)</td>
<td>5(v)</td>
<td></td>
<td>17 (±2)</td>
<td>31 (±4)</td>
<td>+82%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine 10mM</td>
<td>24 (±4)</td>
<td>+41%</td>
<td></td>
<td>24 (±4)</td>
<td>+82%</td>
<td>5(ii)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P</td>
<td>64</td>
<td>5(v)</td>
<td></td>
<td>58 (±6)</td>
<td>108 (±12)</td>
<td>+86%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P + Glucagon 50nM</td>
<td>196 (±18)</td>
<td>+206%</td>
<td></td>
<td>196 (±18)</td>
<td>+206%</td>
<td>5(ii)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P</td>
<td>58</td>
<td>5(vii)</td>
<td></td>
<td>59 (±5)</td>
<td>57 (±7)</td>
<td>-3%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P + Insulin 40uU/ml</td>
<td>58 (±3)</td>
<td>0%</td>
<td></td>
<td>58 (±3)</td>
<td>58 (±3)</td>
<td>0%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P</td>
<td>57</td>
<td>5(ix)</td>
<td></td>
<td>60 (±5)</td>
<td>44 (±5)</td>
<td>-27%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P + Phenformin 2mM</td>
<td>20 (±8)</td>
<td>-65%</td>
<td></td>
<td>20 (±8)</td>
<td>-65%</td>
<td>5(ii)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P</td>
<td>64</td>
<td>5(x)</td>
<td></td>
<td>73 (±8)</td>
<td>57 (±6)</td>
<td>-22%</td>
<td>5(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/P + Wy 23675 2mM</td>
<td>21 (±7)</td>
<td>-67%</td>
<td></td>
<td>21 (±7)</td>
<td>-67%</td>
<td>5(ii)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± the standard error of 4 observations for the perfusions and of 6 observations for the isolated hepatocytes.
The Effects of Insulin, Tolbutamide, Phenformin and Wy 23675 on Glycogenolysis.

Hepatic glucose production involves glycogenolysis as well as gluconeogenesis; thus, the effects of insulin, tolbutamide, phenformin and Wy 23675 on glycogenolysis have been investigated. The basal rate of glucose production from isolated hepatocytes prepared from normal, fed rats was similar to that reported by others with isolated hepatocytes (21, 22, 42) or perfused livers (43).

Glucagon stimulated glycogenolysis in a dose-related manner to more than twice the basal rate of glucose production at $10^{-6}M$ (Table 5(i)). Insulin, however, had no effect on the basal rate of glucose production, but inhibited the stimulation by glucagon (Table 5(i)). Other workers have shown similar effects of glucagon (21, 22, 42, 44) and insulin (22).

The regulation of glycogen metabolism by glucagon and insulin has been the subject of extensive investigation. Both hormones have been shown to affect the levels of cyclic adenosine-3',5'-monophosphate (cAMP) in liver cells (21, 43, 55-59). In muscle, it has been shown that the binding of these hormones to specific membrane sites is accompanied by stimulation of a plasma membrane adenylate cyclase system (61-63). The cAMP produced activates in turn a protein kinase (64, 65), which regulates
metabolism: glycogen phosphorylase and glycogen synthase (66). In the liver, glucagon has been shown to have a glycogenolytic effect (21, 22, 31, 43, 55, 59, 60, 71-74) and this is mimicked by cAMP and its dibutyryl derivative (55) and potentiated by theophylline (55). It has also been shown that, in the perfused liver (60) and isolated hepatocytes (21, 43, 67), glycogenolysis is accompanied by an increase in cAMP levels. It has been suggested that, in the liver, cAMP produced after glucagon administration is responsible for both the activation of glycogen phosphorylase (68) and the inactivation of glycogen synthase (68, 69). Insulin has been shown to oppose both glucagon-stimulated glycogenolysis (31, 41-43, 71, 74, 75) and the glucagon-elevation of cAMP levels (43, 57, 58). Insulin also opposes the glucagon effects on glycogen synthase (69, 70, 76, 77, 79-84) and glycogen phosphorylase (22, 31, 57, 76, 78, 82).

However, the role of cAMP as an intermediary in the actions of glucagon and insulin in the liver is still unclear (58). The activation of liver glycogenolysis and gluconeogenesis by α-adrenergic agonists (59, 85, 86), by vasopressin (87, 88) and by angiotensin (89) appears to be independent of a change in cAMP concentration, although such activation appears to occur at the same enzyme sites as for glucagon. It has been shown that increasing K+ concentration causes inactivation of glycogen phosphorylase and activation of glycogen synthase (90) and that the absence of Ca++ affects the action of several agonists of both gluconeogenesis (91, 92) and phosphorylase activation (93). Van de Werve, Hue and Hers (57) have proposed a role of Ca++ in the effects of glucagon and insulin on phosphorylase in the liver. Changes have been observed in Ca++ concentration (57, 94-96), Ca++ binding to membranes (97, 98) and in mitochondrial Ca++ transport (99) following treatment by these hormones; and Ca++ has been shown to activate phosphorylase kinase (57, 100). Thus, the relationship between these hormones, cAMP and Ca++ and their effects on glycogen metabolism in the liver still has to be fully elucidated.

Neither tolbutamide, phenformin nor Wy 23675 had any effect on basal or glucagon-stimulated glycogenolysis (Table 5(i)).
Steps of Gluconeogenesis.

Gluconeogenesis was investigated in isolated hepatocytes prepared from fasted, normal rats and from fasted, alloxan-diabetic rats. The rates of gluconeogenesis from different substrates with these animals (Table 5(ii).) gave similar values to those reported by others (20, 21, 24, 46, 116, 117). The rate of glucose production declined in the order: 

\[ \text{xylitol} > \text{dihydroxyacetone} > \text{lactate plus pyruvate} > \text{alanine} \]

Figure 5(xii) illustrates the points at which these substrates enter the gluconeogenic pathway and the proposed rate-limiting steps in the pathway (numbered 1, 2 and 3 on the figure - for review, see 159). Thus, these substrates can be used to study the effects of the drugs at the various rate-limiting steps of gluconeogenesis.

The Effects of Glucagon and Insulin on Gluconeogenesis.

Glucagon was shown to stimulate gluconeogenesis from lactate plus pyruvate and from alanine to a greater extent than it did from dihydroxyacetone, while it did not affect gluconeogenesis from xylitol (Table 5(ii).). This indicates that glucagon stimulates gluconeogenesis at the rate-limiting steps marked 2 and 3, not at step 1 (Fig. 5(xii).).

Insulin had no effect on gluconeogenesis from lactate plus pyruvate, but inhibited the submaximal glucagon-stimulation of gluconeogenesis from lactate plus pyruvate (Table 5(ii).). However, this latter effect only became statistically significant at high, non-physiological concentrations of insulin.

Glucagon stimulation of gluconeogenesis in the liver is well-documented (see Table 5(vii) and 27, 51, 60, 111, 113, 124, 127, 134, 135, 158, 160, 161, 163, 164, 171, 175, 203, 204, 207, 215, 249, 250), but there is still no agreement about its site of action. Different gluconeogenic substrates have been used in attempts to isolate the rate-limiting step(s) at which glucagon acts (see Table 5(vii).). As can be seen, different workers have found considerable variation in the degree of stimulation of gluconeogenesis.
Diagram to illustrate the points of entrance of substrates into the gluconeogenic pathway.

GLUCOSE

Gluco kinase (1) Glucose-6-phosphatase

GLUCOSE-6-PHOSPHATE

XYLITOL

D-XYLOLose

FRUCTOSE-6-PHOSPHATE

Phosphofructokinase (2) Fructose bisphosphatase

FRUCTOSE BISPHOSPHATE

D-XYLOLose-5-P

DIHYDROXYACETONE

DIHYDROXYACETONE PHOSPHATE

LACTATE

Lactate dehydrogenase

PHOSPHOENOLPYRUVATE

PEP carboxykinase

OXALOACETATE

PYRUVATE

Pyruvate kinase

(3) Pyruvate carboxylase

ALANINE

CITRATE

KREBS CYCLE

NB: Effects of metabolic compartmentation of the reactions in the pathway have been omitted for clarity.

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EFFECT OF GLUCAGON ON GLUCONEOGENESIS FROM SELECTED SUBSTRATES BY FASTED RAT LIVERS.

<table>
<thead>
<tr>
<th>Alanine</th>
<th>Lactate and/or Pyruvate</th>
<th>Dihydroxyacetone</th>
<th>Fructose</th>
<th>Xylitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>45% (21)</td>
<td>75% (4)</td>
<td>31% (21)</td>
<td>32% (21)</td>
<td>18% (46)</td>
</tr>
<tr>
<td>38% (46)</td>
<td>60% (21)</td>
<td>21% (25)</td>
<td>20% (23)</td>
<td>15% (91)</td>
</tr>
<tr>
<td>37% (71)</td>
<td>227% (23)</td>
<td>58% (46)</td>
<td>21% (25)</td>
<td>22% (112)</td>
</tr>
<tr>
<td>42% (91)</td>
<td>145% (25)</td>
<td>100% (49)</td>
<td>59% (46)</td>
<td></td>
</tr>
<tr>
<td>50% (128)</td>
<td>222% (29)</td>
<td>50% (74)</td>
<td>20% (91)</td>
<td></td>
</tr>
<tr>
<td>19% (217)</td>
<td>134% (35)</td>
<td>37% (112)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% (253)</td>
<td>40% (44)</td>
<td>50% (116)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33% (46)</td>
<td>34% (230)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24% (48)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>57% (71)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>56% (91)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51% (101)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>78% (112)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>71% (121)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>44% (138)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>89% (141)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30% (214)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21% (217)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ \bar{x} = 40\%\]
\[ \bar{x} = 81\%\]
\[ \bar{x} = 48\%\]
\[ \bar{x} = 33\%\]
\[ \bar{x} = 18\%\]

(NB: Figures in parenthesis indicate references)
is general agreement that such a stimulation does occur. Their findings have pointed to effects of glucagon on rate-limiting steps 2 and 3 in Figure 5(xii).

The effects of glucagon on the enzymes in these rate-limiting steps have been extensively investigated. It has been shown that glucagon stimulates pyruvate carboxylase (4, 51, 121, 128, 255) and phosphoenolpyruvate carboxykinase (25, 51) and inhibits pyruvate kinase (46, 48, 49, 51, 114, 135-138, 175-177, 183, 202-204, 215, 216, 220, 221, 258, 259), while having no effect on pyruvate dehydrogenase (217) at step 3. At step 2, glucagon stimulates fructose bisphosphatase (51, 112, 114, 120, 121, 134, 220, 227, 228, 258, 259) and inhibits phosphofructokinase (51, 112, 114-116, 120, 121, 134, 216, 218, 219, 220, 227, 228, 230, 258, 259, 272, 273). All of these effects of glucagon would tend to restrict glycolysis and to increase gluconeogenesis.

Despite considerable research, the primary site of glucagon action remains unknown. It was thought that glucagon acted only on the conversion of pyruvate to phosphoenolpyruvate (25, 60, 158, 159), but more recent studies (49, 91, 111, 112, 124, 134, 160, 171) have shown that gluconeogenesis from dihydroxyacetone, fructose, xylitol, and glycerol, which enter the gluconeogenic pathway above phosphoenolpyruvate, is also stimulated. There is the possibility that glucagon stimulates gluconeogenesis from these substrates by converting lactate formed by glycolysis to glucose. However, quinolinate has been used to inhibit phosphoenolpyruvate carboxykinase and glucagon still stimulated glucose production from dihydroxyacetone (111, 112), indicating that the effect did not depend on the resynthesis of phosphoenolpyruvate from pyruvate. It has also been shown that inhibitors of steps from lactate to dihydroxyacetone phosphate have no effect on gluconeogenesis from dihydroxyacetone (i.e., using \( \beta \)-phenylpyruvate to inhibit pyruvate carboxylase; \( \alpha \)-cyanocinnamate to inhibit pyruvate transport; hydrazine and tryptophan to inhibit phosphoenolpyruvate carboxykinase; \( n \)-butylmalonate to inhibit malate transport; and amino-oxyacetate to inhibit transamination (49).]
134,175) and it has been proposed that this could account for most of the increase seen in gluconeogenesis from dihydroxyacetone and the cross-over between pyruvate and phosphoenolpyruvate (49), indicating pyruvate kinase as a site of glucagon action. In support of this concept, is the finding that glucagon loses its ability to stimulate gluconeogenesis from dihydroxyacetone during periods of prolonged starvation (49), when pyruvate kinase activity is already low (172-174). It has been reported that 80% of phosphoenolpyruvate formed from pyruvate is reconverted to pyruvate via pyruvate kinase in the liver from fed rats, whilst only 30% is recycled in liver from starved rats (183). Thus, an effect of glucagon that diminished recycling of phosphoenolpyruvate to pyruvate could be very important in gluconeogenesis from physiological substrates. Not surprisingly, pyruvate kinase has been proposed as a primary site of glucagon action (135,175). However, van Berkel et al (226) have shown that glucagon regulation of pyruvate kinase is relatively insensitive to changes in the extracellular environment (such as the concentrations of lactate, glucose, extracellular Ca++, Na+ or K+), whereas these factors do influence the effects of glucagon on gluconeogenesis. They, therefore, concluded that glucagon action on pyruvate kinase is not the only site of its regulation of gluconeogenesis.

Liver pyruvate kinase can be phosphorylated and simultaneously inactivated by a cAMP-dependant protein kinase (181,229,275): glucagon has also been shown to phosphorylate and inactivate pyruvate kinase as well as to cause a decrease in the apparent flux through pyruvate kinase (213,275,276). It, therefore, appears likely that the glucagon effects on pyruvate kinase are mediated by cAMP.

Recent studies with isotopes have suggested that glucagon affects gluconeogenesis at the phosphofructokinase/fructose bisphosphatase cycle (134,227) as well as the phosphoenolpyruvate cycles, involving pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase (175, 205). It has, thus, been suggested that the primary site of glucagon
bisphosphatase cycle (49,112,134,228) and that the regulation of the
phosphoenolpyruvate cycle is a result of changes in fructose bisphosphate
levels, an allosteric activator of pyruvate kinase (178-180).

It has also been proposed that glucagon could affect gluconeogenesis
by stimulating the transport of pyruvate into mitochondria (101,108,147,
287). Pyruvate enters the mitochondria on a specific carrier (130,133,
278-285), a net flux of pyruvate occurring with a H⁺ (285). Mitochondria
isolated from livers of rats pretreated with glucagon exhibited enhanced
rates of pyruvate metabolism (108); this effect is also observed with
hepatocytes incubated with glucagon or dibutyryl cAMP (286). Glucagon
has been shown to cause enhanced rates of pyruvate transport (133,147,287),
the effect being mediated by an increase in matrix pH, which in turn
stimulates pyruvate transport (287).

Glucagon activation of gluconeogenesis has also been attributed to its
lipolytic action (121,129,154-157,161,207,208). Glucagon has been shown
to elevate matrix acetyl CoA (142-145), which is the end product of fatty
acid oxidation and is an obligatory activator of pyruvate carboxylase
(142-145,162). Tissue concentration of long-chain acyl CoA has been
suggested as a factor which could affect the major pathways of glucose
metabolism (188). The concentration of long chain acyl CoA in the liver
has been shown to coincide with different states of intermediary
glucose metabolism, being increased in diabetes and after prolonged
starvation and decreased after restoration of insulin or glucose
refeeding (188,192,193). The liver concentration of long chain acyl CoA has
been shown to be increased by increasing circulating oleic acid
(129,188); the latter depends upon the circulating concentrations of
glucagon and insulin (188). It has been shown that several long chain
acyl CoA derivatives inhibit the translocation of adenine nucleotides
through the inner mitochondrial membrane (194), thus affecting the
ATP/ADP ratio, which affects gluconeogenesis (195,196), pyruvate conversion
to acetyl CoA (196,197) and citric acid cycle activity (198). An
presence of oleic acid and glucagon (71,161,163). Also, like oleic acid, glucagon increases the redox state (51,127,128,145,146) and an increase in the matrix ATP/ADP ratio can activate pyruvate carboxylase (145,146). However, most workers (51,139-141,158,164,206,274) now agree that glucagon does not stimulate gluconeogenesis by merely increasing fatty acid supply. It has been shown that glycodiazine, an inhibitor of lipolysis (209), does not prevent the glucagon stimulation of gluconeogenesis (141). Also, studies on the effects of glucagon and oleate on the metabolic compartmentation in the liver (51), have revealed marked differences in their effects on cellular concentrations of glutamate, 2-oxoglutarate and phosphoenolpyruvate; and oleate in the presence of lactate stimulates citrate formation, while glucagon does not (51).

It seems unlikely that any one of the above effects of glucagon can explain its stimulation of gluconeogenesis; probably, glucagon achieves its effect by a concerted mechanism involving some or all of the above actions.

There appears to be a general agreement that cAMP acts as a mediator of the glucagon-stimulation of gluconeogenesis (23,25,44,277). It has been shown that glucagon elevates cAMP levels in the hepatocyte (21,33,43,60,71,101,114,184,273) and stimulates adenyl cyclase (168,185). Also, cAMP and its analogs have been shown to mimic many of the effects of glucagon (21,23,25,44,60,101,110,112,114-116,124,134,135,138,158,175,176,203,204,216,218,219,229,273).

Insulin has been shown to have no effect on basal rates of gluconeogenesis (36,37,49,165), but it does inhibit glucagon-stimulated gluconeogenesis (31,33,34,38,43,49,58,71,128,165,243) and cAMP-stimulated gluconeogenesis (165,167).

Insulin appears to act by opposing the actions of glucagon. For example, insulin reverses the glucagon inhibition of pyruvate kinase (138), but has negligible effects on pyruvate kinase in the absence of glucagon (138). Insulin may also prevent the glucagon-stimulation of pyruvate.
redox state (127,128). Insulin is an antilipolytic agent and can inhibit glucagon-stimulation of lipolysis (288-291). By opposing these actions of glucagon, many of which are thought to contribute to the glucagon-stimulation of gluconeogenesis, insulin is able to exert a 'moderating' influence on the glucagon effect.

The actions of glucagon are thought to be mediated by cAMP: insulin lowers cAMP levels (33,43,158,166). Insulin has been shown to inhibit adenyl cyclase (168,247,248) and to stimulate cAMP-phosphodiesterase (169, 211,212,245,246,252). However, insulin has been shown to inhibit cAMP-stimulated gluconeogenesis (165,167) and it has been suggested that it might act by inhibiting cAMP at the level of protein kinase (244).

The Effects of Tolbutamide and Phenformin on Gluconeogenesis.

In this work, tolbutamide had no effect on either basal rates of gluconeogenesis or on glucagon-stimulated gluconeogenesis (Table 5(ii)). As mentioned in the introduction to this thesis, various effects of sulphonylureas have been reported on glucose metabolism in the liver, including some inhibition of gluconeogenesis in diabetic subjects (292). However, most of the hepatic effects of the sulphonylureas are thought to require the presence of insulin (293).

Phenformin was shown to inhibit gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but had no effect on gluconeogenesis from xylitol (Table 5(ii)). However, these inhibitory effects were only seen with high concentrations of phenformin. When gluconeogenesis was stimulated by glucagon, the concentration of phenformin required to produce an inhibitory effect was considerably lowered (Table 5(ii)).

Inhibition by phenformin of gluconeogenesis in the liver is well-documented (32,39,101,104,105,231-234,257,261,262,265; for further references, see also the Introduction to this thesis). Much of this work is with concentrations of phenformin that (i) are not attained during therapeutic use of the drug and (ii) inhibit mitochondrial respiration.
of phenformin that can be achieved with therapeutic administration (101, 105, 294) and which are too low to be associated with inhibition of mitochondrial respiration. It has also been shown that phenformin can inhibit gluconeogenesis when it is stimulated by glucagon (101) or by diabetes (257, 265) at lower concentrations than those necessary to inhibit basal rates of gluconeogenesis. These findings are in agreement with the results reported in this chapter.

Phenformin is without effect on many of the enzymes of gluconeogenesis (unless given at high concentrations) - for example, it does not affect fructose bisphosphatase (262), phosphofructokinase (263) or the conversion of 3-phosphoglycerate to glyceraldehyde-3-phosphate at concentrations up to 8 mM (261). However, at 47 mM phenformin did inhibit 3-phosphoglycerate kinase by 50% (264). At concentrations up to 0.8 mM, it had no effect on pyruvate carboxylase (105) and up to 50 mM had no effect on enolase (264). Thus, the effects of phenformin on gluconeogenesis do not appear to be due to a direct effect on the enzymes of gluconeogenesis.

For reasons discussed in detail in the Introduction to this thesis, the effect of phenformin on gluconeogenesis does not seem to be related directly to the energy requirements of gluconeogenesis. The membrane-biguanide interaction mechanisms proposed by Schafer (267, 268) and the enhancement of Ca++-uptake into mitochondria by biguanides proposed by Davidoff (269) appear to offer the most likely possibilities for the subcellular site of biguanide action. The evidence for these theories is discussed in the Introduction to this thesis.

The Effect of Wy 23675 on Gluconeogenesis.

Wy 23675 exhibited similar effects to phenformin on gluconeogenesis in hepatocytes prepared from normal starved rats in the results reported in this chapter (Table 5(iii)). Wy 23675 inhibited gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but had no effects on gluconeogenesis from xylitol. When gluconeogenesis was stimulated by

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for inhibitory effects on basal rates of gluconeogenesis.

The possibility for an effect of Wy 23675 on pyruvate dehydrogenase was mentioned in Chapters 3 and 4 of this thesis. This raises the question of whether such an activation of pyruvate dehydrogenase in the liver cell could affect gluconeogenesis as observed in the results reported in this chapter. Theoretically, an activation of pyruvate dehydrogenase could divert the supply of lactate and pyruvate from gluconeogenesis through pyruvate carboxylase to the formation of acetyl CoA. Could this also account for the observed inhibition of gluconeogenesis from dihydroxycetone? This seems unlikely as dihydroxycetone enters the gluconeogenic pathway at the level of the triose phosphates. Also, Wy 23675 is most potent at inhibiting gluconeogenesis when it is stimulated by glucagon and therefore under circumstances where very little glycolysis occurs. Thus, Wy 23675 is unlikely to be inhibiting gluconeogenesis from dihydroxycetone by preventing any pyruvate formed by glycolysis from re-entering the gluconeogenic pathway by diverting it through pyruvate dehydrogenase.

There is also other evidence which argues against activation of pyruvate dehydrogenase being involved in inhibition of gluconeogenesis. Dichloroacetate is a potent activator of pyruvate dehydrogenase (295,296) and, under certain circumstances, it can inhibit gluconeogenesis (297,298). However, this inhibition is only seen in the presence of a low-bicarbonate medium and not in Krebs-Henseleit; therefore, these effects on gluconeogenesis are probably not important in vivo (299). Wy 23675 inhibits gluconeogenesis in a Krebs-bicarbonate medium, so it is unlikely to be acting through the same mechanism as dichloroacetate. Also, even under circumstances where dichloroacetate did inhibit gluconeogenesis it only did so from pyruvate, lactate and alanine, and not from glycerol, which enters the gluconeogenic pathway at the level of the triose phosphates (300). This is a further difference between the observed effects of activation of pyruvate dehydrogenase and those seen in the
One of the arguments presented in the introduction in this thesis against the likelihood of phenformin affecting gluconeogenesis by inhibiting mitochondrial respiration was that it inhibited the rate of glucoheogenesis from lactate plus pyruvate and dihydroxyacetone to the same extent and had no effect on gluconeogenesis from xylitol, despite the fact that more high energy phosphate is required for glucose synthesis from lactate than from dihydroxyacetone and, also, that some high energy phosphate is required for glucose synthesis from xylitol. The same argument can be applied to the results with Wy 23675, so it is unlikely that it is inhibiting gluconeogenesis by affecting the energy supply.

It has been suggested that glucagon stimulates gluconeogenesis by inhibiting pyruvate kinase (135, 175) and also that changes in pyruvate kinase activity are brought about by changes in fructose bisphosphate levels through effects of glucagon on the phosphofructokinase/fructose bisphosphatase cycle (49, 112, 134, 228). It is possible that Wy 23675 is also acting at these levels to inhibit gluconeogenesis by inhibiting fructose bisphosphatase and/or stimulating phosphofructokinase, leading to an increase in fructose bisphosphate and an activation of pyruvate kinase. Such an effect could explain why Wy 23675 affects gluconeogenesis from both lactate plus pyruvate and from dihydroxyacetone and not from xylitol; and also why lower concentrations of Wy 23675 are required for inhibitory effects when gluconeogenesis is stimulated by glucagon.

The Effects of Insulin, Tolbutamide, Phenformin and Wy 23675 on Gluconeogenesis in Alloxan-Diabetic Rats.

Alloxan-diabetic rats were shown to have significantly higher rates of gluconeogenesis from lactate plus pyruvate, from dihydroxyacetone and from xylitol than normal rats [Table 5(iii)]. This effect has been established by many other researchers (158, 256, 301-305). Glucagon was able to further stimulate gluconeogenesis from lactate plus pyruvate [Table 5(iv)], but this effect was only seen with concentrations of
gluconeogenesis in the normal, fasted rats and, even then, the degree of stimulation was relatively small: this is a reflection of the near-maximal rates of gluconeogenesis that were occurring in these diabetic rats.

The lack of insulin has been shown to result in an elevated cAMP level in the liver (43, 184, 251) and an elevated rate of gluconeogenesis; insulin therapy reverses these effects (256, 305, 306). In the results reported in this chapter, insulin had no acute effect on gluconeogenesis from lactate and pyruvate in hepatocytes prepared from alloxan-diabetic rats (Table 5(iv)), but did inhibit gluconeogenesis when it was administered in vivo for several days prior to the preparation of the hepatocytes (Table 5(v)).

Tolbutamide had no effect on gluconeogenesis in hepatocytes prepared from alloxan-diabetic rats either when it was administered in vitro or in vivo (Tables 5(iv) and 5(v)). Phenformin inhibited gluconeogenesis in hepatocytes from alloxan-diabetic rats in concentrations 100-fold lower than those required for inhibition of basal rates in hepatocytes from normal rats (Table 5(iv)). Other researchers have shown similar effects (257, 265).

Wy 23675 had similar effects to phenformin (Table 5(iv)), requiring a lower concentration of the drug to inhibit gluconeogenesis in hepatocytes prepared from alloxan-diabetic rats than in those prepared from normal rats. These effects correspond to those seen in normal hepatocytes when gluconeogenesis is stimulated by glucagon.
75. Haft, D.E. Diabetes 17, 244-250, 1968.
Metabolic Studies* (Eds. Tager, J.M., Soling, H.D. & Williamson, J.R.),
444-447, North-Holland/american Elsevier, Amsterdam, Oxford and N.Y.,
1976.

137. Pilkis, S.J., Claus, T.H., Ricou, J.P. & Park, C.R. J. Biol. Chem. 251,
1355-1360, 1976.

3756-3762, 1976.

139. Ross, B.D., Hems, R., Freedland, R.A. & Krebs, H.A. Biochem. J. 105,


148. Haynes, R.C., Jr. in 'Energy Metabolism and the Regulation of Metabolic
Processes in Mitochondria' (Eds. Mehlman, M.A. & Hanson, R.W.), 239-252,


151. Pogell, B.M., Taketa, K. & Sarngadharan, M.G. J. Biol. Chem. 246,

152. Carlson, C.W., Baxter, R.C., Ulm, E.H. & Pogell, B.M. J. Biol. Chem. 248,


1966.


Chapter 6
Chapter 6.

THE EFFECTS OF PHENFORMIN AND WY 23675 ON HEPATIC METABOLITE LEVELS.

1. Summary.................................................................................................................. 238
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This chapter contains measurements of hepatic metabolite levels in untreated, alloxan-diabetic rats compared to alloxan-diabetic rats after seven days administration of phenformin or Wy 23675 in vivo. Following alloxan-treatment, the body weights of the rats in the control group fell, while those in the phenformin-treated group remained constant and those in the Wy 23675-treated group increased. Phenformin treatment resulted in a fall in blood glucose levels compared with the controls and a doubling of the blood lactate/pyruvate ratio, while Wy 23675 treatment resulted in a greater fall in blood glucose than phenformin without any change in the lactate/pyruvate ratio.

The pattern of hepatic metabolite levels following phenformin treatment showed a negative cross-over between 3-phosphoglycerate and dihydroxyacetone phosphate. Measurement of appropriate metabolite ratios showed a more reduced state in the cytosol and mitochondria following phenformin treatment and these redox changes are discussed.

Wy 23675 treatment showed a negative cross-over in hepatic metabolite levels between malate and phosphoenolpyruvate, indicating a possible effect of this drug on the enzyme phosphoenolpyruvate carboxykinase (PEPCK). However, there was no increase in lactate levels, and this is discussed in comparison with a known inhibitor of PEPCK, 3-mercaptopicolinate, which causes marked increases in lactate levels.

A further experiment described in this chapter failed to show any direct effect of Wy 23675 in vitro on PEPCK, while 3-mercaptopicolinate inhibited by 87% at 1mM. The possibility that differences in the experimental conditions, which were employed to measure PEPCK and those used in the experiments to measure hepatic metabolite levels, could explain the lack of any effect of Wy 23675 on the enzyme is discussed together with possible alternative modes of action of Wy 23675 on gluconeogenesis.
In chapter 5, phenformin and Wy 23675 were both shown to inhibit gluconeogenesis from lactate plus pyruvate and from dihydroxyacetone, but to have no effects on gluconeogenesis from xylitol. These results indicated effects of these compounds (either direct or indirect) on the rate-limiting steps in gluconeogenesis involving the enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase (controlling the conversion of pyruvate to phosphoenolpyruvate) and by fructose bisphosphate and phosphofructokinase (controlling the conversion of fructose bisphosphate to fructose-6-phosphate).

In this chapter, the effects of phenformin and Wy 23675 have been investigated on selected metabolite levels in the livers of alloxan-diabetic rats, following seven days treatment with the drugs in vivo. This approach has been used extensively to determine the sites of action of many hormones and drugs on glucose metabolism in the liver (1-24).

The results of this experiment indicated a possible effect of Wy 23675 on the enzyme phosphoenolpyruvate carboxykinase (PEPCK). This possibility has been further investigated by measuring the effect of Wy 23675 in vitro on rat liver cytosolic PEPCK activity.
Materials

The sources of all drugs used were given in earlier chapters. All enzymes, chemicals and reagents were obtained from Sigma, Kingston-upon-Thames, Surrey, with the exception of hydrazine hydrate, which was obtained from Koch-Light, Colnbrook, Buckinghamshire.

1. Methods For Measurements of Hepatic Metabolite Levels.

Treatment of Animals.

The rats used were male Charles River CD strain (180-220gms), maintained as described in Chapter 1. They were made alloxan-diabetic by treatment with alloxan monohydrate (0.2mmol/kg i.p.), following a 24-hr fast. Blood samples were collected from the tail vein 48 hours after alloxan-treatment and rats with fed blood glucose levels in excess of 20 mmol/l. were randomized into 3 groups of 6 rats for the purposes of this study. Each group was then orally dosed twice daily (at 0800 and 2000 hrs) with either CMC (Group 1), phenformin (0.2mmol/kg) (Group 2), or Wy 23675 (0.2mmol/kg) (Group 3) for 7 days. Rats were bled immediately prior to drug treatment and after 4 days of treatment; the blood samples were analyzed for glucose (see Chapter 1), lactate and pyruvate (Sigma Kit No's 826-UV and 726-UV, respectively).

On day 8, the rats were orally dosed in the morning and killed 1 hr. afterwards by cervical dislocation. The livers were removed and freeze-clamped within 15 seconds, by pressing them between plates of aluminium tongs, which had been previously placed in liquid nitrogen. The frozen livers were then individually powdered in a Teflon percussion mortar cooled in dry ice.

Preparation of Acid Extracts.

An acid extract was made from each powdered liver. Aliquots of the powdered livers were placed in pre-weighed centrifuge tubes stored in powdered dry ice. The tubes were weighed after addition of the powered liver to determine the weight of the aliquot added. 3.5-volumes of 8% (v/v) HClO₄ made up in 40% (v/v) ethanol was added to the powdered...
using a Teflon pestle. The homogenate was decanted into glass centrifuge tubes and was centrifuged at 25,000g, for 10 minutes at -10°C. The supernatant was decanted into graduated centrifuge tubes and stored in dry ice, while any precipitate remaining in the homogenizing tubes was transferred to the pellet in the appropriate centrifuge tube by washing with 2.5-volumes of 6% (v/v) HClO₄. This was then mixed to a smooth paste with a teflon rod and centrifuged at 25,000g, for 10 minutes at -10°C. The two supernatants thus obtained from each liver homogenate were combined and the volume recorded. The pH was then adjusted to 5.5 - 6.0 by slow addition of 3M K₂CO₃ containing 0.5M triethanolamine base, mixing continuously, and the total volume recorded. After recentrifugation to remove the precipitated KClO₄, the final supernatants were stored at -20°C. The number of grams of powdered liver was calculated per 100 mls of acid extract and recorded for each liver (the average was 171 gms/1000 mls).

Measurement of Metabolites.

The following metabolites were measured for each liver extract: - Glucose (Glc); Glucose-6-phosphate (G6P); Fructose-6-phosphate (F6P); Fructose bisphosphate (FBP); Dihydroxyacetone-phosphate (DHAP); Glyceraldehyde-3-phosphate (GLAP); 3-Phosphoglycerate (3PG); 2-Phosphoglycerate (2PG); Phosphoenolpyruvate (PEP); Pyruvate (Pyr); Lactate (Lac); Oxaloacetate (OAA); Citrate (Cit); Isocitrate (Isocit); 2-Oxoglutarate (2OG); Glutamate (Glut); Malate (Mal); Acetyl CoA (AcCoA); 3-Hydroxybutyrate (3OH-B); Acetoacetate (AcAc); Adenosine triphosphate (ATP); Adenosine diphosphate (ADP); Adenosine monophosphate (AMP).

All the metabolites were measured by the methods published by Bergmeyer (38), except for lactate and pyruvate (Sigma kits 826-UV and 726-UV) and glucose (Autoanalyser - see Chapter 1). The metabolites were measured in Sarstedt plastic disposable cuvettes, using a Cecil CE 292 ultraviolet spectrophotometer. The concentrations were determined by measuring the change in optical density of reduced pyridine nucleotides at 340 nM in the enzyme tests. The results were expressed in μmoles/gm. of wet liver weight.

The methods described here have been developed at Glaxo Research (Ware) Ltd., Priory Street, Ware, Hertfordshire from previously published assays.

1. Preparation of the Enzyme (39).

A crude PEPCK preparation was produced as follows (the procedure was carried out at 4°C):

48-hour fasted, normal female rats (weight range 180-220gms) were killed by cervical dislocation and their livers removed and rinsed in ice-cold saline (0.9% NaCl). Following mincing, a 1:3 (w/v) homogenate was prepared in 0.25M sucrose containing 10mM triethanolamine chloride (pH 8.1), using an 'Ultraturex' homogeniser.

The homogenate was then aliquoted into Eppendorf centrifuge tubes and centrifuged in an Eppendorf 3200 bench centrifuge at approximately 12000g for 10 minutes. The resultant supernatant comprised the cytosolic fraction of the homogenate. This was decanted and stored on ice until used (for up to 4 hours).

2. Pre-incubation of Cytosolic Preparation with Test Compounds.

The compounds under investigation were pre-incubated with the cytosol preparation in the presence of FeCl₂ for 10 minutes at 0°C. The final incubation volume was 1 ml, made up as follows:

0.2 mls cytosol preparation
0.3 mls 14mM HEPES/NaOH, pH 8.1, containing 2.7mM dithiothreitol (giving final concentrations of 4.2mM and 0.8mM, respectively).
0.1 mls 1mM FeCl₂ (giving final concentration of 0.1mM).
0.4 mls 2.5mM test compound or control (giving 1mM final concentration)

At the end of the 10 minute period, 0.2 mls of this pre-incubation mixture was transferred into tubes containing the other constituents for the PEPCK assay (see PEPCK assay, Part A).
PEPCK activity was monitored in the physiological direction of phosphoenolpyruvate (PEP) synthesis from oxaloacetate (OAA). The assay comprised two parts: PEP generated by the activity of PEPCK was removed from a deproteinized assay mixture and measured using a linked, spectrophotometric assay. This involved the conversion of PEP to pyruvate and lactate with a concomitant reduction in optical density due to NADH oxidation:

\[
\text{OAA} \xrightarrow{\text{PEPCK}} \text{PEP} \xrightarrow{\text{PK}} \text{Pyruvate} \xrightarrow{\text{LDH}} \text{Lactate}
\]

(\text{ITP = Inosine-5'-triphosphate ; GTP = Guanosine-5'-triphosphate})
(\text{PK = Pyruvate kinase ; LDH = Lactate dehydrogenase})

Since 1 mole of PEP causes the oxidation of 1 mole of NADH, the assay effectively measured PEP concentration.


The final 1 ml reaction mixture, pH 8.1, was made up as follows:

0.2 ml pre-incubation mixture
0.1 ml 80mM OAA (adjusted to pH 7.0) (Final concentration: 8mM OAA)
0.7 ml 143mM Tris-HCl, pH 8.1, containing 25.7mM MgCl₂ and 8.6mM Na₂ITP (Final concentrations: 100mM Tris-HCl, 18mM MgCl₂, 6mM Na₂ITP)

After a 5 minute temperature equilibration period at 37°C, the reaction was initiated by the addition of the 0.2 ml of pre-incubation mixture. The reaction was terminated by the addition of 0.1 ml of a 50mg KBH₄/1 ml 1mM NaOH after 10 minutes at 37°C. (The KBH₄/NaOH solution reduced unreacted OAA to malate, thereby preventing spontaneous decarboxylation to pyruvate). After 5 minutes on ice, excess KBH₄ was removed by acidification with 0.2 ml 20% (w/v) HClO₄ followed by vortex mixing. Following neutralization with 0.16 ml 0.5M triethanolamine-HCl, pH 7.4/2M KOH (Final pH = 13-14), the samples were centrifuged in an Eppendorf 3200 for 2 mins.

This assay was run at room temperature at pH 7.6. The final 1 ml reaction volume was made up as follows:

- 0.1 mls deproteinized sample from Part A
- 0.1 mls (40 units/ml) pyruvate kinase (Final concentration: 4 units/ml)
- 0.8 mls 87.5 mM Tris-Cl, pH 7.6, containing 5.25 mM MgSO$_4$, 41.25 mM KCl, 0.15 mM NADH, 2.13 mM Na$_2$ADP and 6.9 units LDH (Final concentrations: 70 mM Tris-Cl, 4.2 mM MgSO$_4$, 33 mM KCl, 0.12 mM NADH, 1.7 mM Na$_2$ADP, 5.5 units/ml LDH)

The initial optical density was measured at 340 nM before the pyruvate kinase was added to the reaction mixture (this permitted residual pyruvate to be reduced to lactate and thus prevented it from interfering with the estimation of PEP concentration). The pyruvate kinase was then added to the assay mixture and 25 minutes later a second optical density was measured; the optical density change was then calculated.


PEPCK activity is expressed as $\mu$moles PEP produced per minute per ml of cytosol and is calculated from the equation:

$$\frac{\Delta OD \times V_1 \times 36.5}{\xi \times V_2 \times t}$$

where $\Delta OD$ = the change in optical density at 340 nM
V1 = Volume of assay Part B mixture (ie: 1 ml)
36.5 = Multiplication factor to allow for dilutions,
$\xi$ = millimolar extinction coefficient of NADH (ie: 6.22)
V2 = Volume of assay Part A taken for PEP determination (0.1 ml)
t = Reaction time of Part A of the assay (ie: 10 minutes)

Therefore $\mu$moles PEP produced/min/ml cytosol = $\Delta OD \times 5.868$

5. Experimental Controls.

Blanks were run containing 0.2 mls sucrose buffer instead of 0.2 mls cytosol. Controls were run containing 0.4 mls water and a positive control was run with 0.4 mls 3-mercaptopicolinate to give 1 mM final concentration [3-mercaptopicolinate is a known inhibitor of PEPCK, (13, 14, 22, 42)].
1. Measurement of Hepatic Metabolite Levels.


The body weights of the rats in the control group fell by a mean of 9.7% by the end of seven days dosing with CMC, while those for the phenformin-treated group had virtually no change and those for the Wy 23675-treated group increased by a mean of 8.6% [Table 6(i)].

It was shown that after four days drug treatment the blood glucose levels of the control group rose by a mean of 37.6%, while that of the phenformin-treated group fell by a mean of 21.5% and that of the Wy 23675-treated group fell by a mean of 33.7% [Table 6(ii)]. During the same period, it was shown that the lactate and pyruvate levels remained unaltered in both the control and the Wy 23675-treated groups, while the phenformin-treated group showed a marked elevation in lactate levels (the mean rising by 220%), but no change in pyruvate levels. Thus, the phenformin-treated group showed an increase in the lactate/pyruvate ratio [Table 6(iii)].

b). Liver Metabolite Levels.

The liver metabolite levels are summarized in Table 6(iv) and Figure 6(i). In the phenformin-treated group, there were significant reductions in the levels of glucose, fructose bisphosphate, dihydroxyacetone phosphate, citrate, acetoacetate and adenosine triphosphate. There were also significant increases in the levels of 3-phosphoglycerate, phosphoenolpyruvate, lactate, glutamate, acetyl CoA, 3-hydroxybutyrate and adenosine diphosphate. The ratios of lactate/pyruvate, glutamate/2-oxoglutarate, and 3-hydroxybutyrate/acetoacetate were markedly elevated, while the ratio of ATP/ADP was reduced.

In the Wy 23675-treated group, there were significant reductions in the levels of glucose, glucose-6-phosphate, fructose-6-phosphate, fructose bisphosphate, dihydroxyacetone phosphate and phosphoenolpyruvate. There were also significant increases in the levels of citrate and malate. There were no changes in the ratios of lactate/pyruvate, glutamate/

3-mercaptopicolinate, a known inhibitor of phosphoenolpyruvate carboxykinase (13, 14, 22, 42), inhibited the enzyme by 87% at 1mM concentration, while Wy 23675 had no significant effect at either 0.1mM or 1mM. The details of these results are shown in Table 6(v).
THE EFFECTS OF PHENFORMIN AND WY 23675 ON HEPATIC METABOLITE LEVELS.

Chapter 6.

Figure No. Title
6(i) Cross-over Plots of Metabolite Levels in Liver After Seven Days Drug Treatment.

Table No. Title
6(i) Rat Body Weights and Weight of Powdered Liver in the Extract.
6(ii) Blood Glucose Levels Before and After Four Days of Drug Treatment.
6(iii) Blood Lactate and Pyruvate Levels Before and After Four Days of Drug Treatment.
6(iv) Summary of Metabolite Levels in Liver After Seven Days Drug Treatment.
6(v) The Effect of Wy 23675 on the Enzyme Phosphoenolpyruvate Carboxykinase.
For practical details, see text.
Statistical significance was determined by Student's t-Test:
* \( p < 0.05 \)
** \( p < 0.01 \)
*** \( p < 0.001 \)

<table>
<thead>
<tr>
<th>Group Treatment</th>
<th>Rat Body Weights (gms)</th>
<th>Weight of Powdered Liver in Acid Extract (gms/1000 mls)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to Drug Treatment</td>
<td>At Sacrifice</td>
</tr>
<tr>
<td>1. CMC Control</td>
<td>217 203</td>
<td>164 176</td>
</tr>
<tr>
<td></td>
<td>210 217</td>
<td>153 229</td>
</tr>
<tr>
<td></td>
<td>195 210</td>
<td>196 212</td>
</tr>
<tr>
<td></td>
<td>217 210</td>
<td>164.5</td>
</tr>
<tr>
<td>Mean ( \pm ) SEM</td>
<td>208.7 ( \pm ) 3.5</td>
<td>188.3 ( \pm ) 11.9</td>
</tr>
<tr>
<td>2. Phenformin (0.2 \text{mmol/kg}, \text{twice daily})</td>
<td>199 205 206 186 211 208</td>
<td>214 236 200 176 204 194</td>
</tr>
<tr>
<td>Mean ( \pm ) SEM</td>
<td>202.5 ( \pm ) 3.7</td>
<td>204.0 ( \pm ) 8.2</td>
</tr>
<tr>
<td>3. Wy 23675 (0.2 \text{mmol/kg}, \text{twice daily})</td>
<td>184 199 200 191 211</td>
<td>224 184 207 232 229</td>
</tr>
<tr>
<td>Mean ( \pm ) SEM</td>
<td>200.7 ( \pm ) 5.2</td>
<td>217.8 ( \pm ) 7.7</td>
</tr>
</tbody>
</table>
For practical details, see text.
Statistical significance was determined by Student's t-Test:
* p<0.05 ; ** p<0.01 ; *** p<0.001

<table>
<thead>
<tr>
<th>Group Treatment</th>
<th>Blood Glucose Levels (mmol/l.)</th>
<th>Difference (2 - 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to Drug Treatment</td>
<td>After 4 Days Drug Treatment</td>
</tr>
<tr>
<td>1. Prior to Drug Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td>29.04</td>
<td>39.60</td>
</tr>
<tr>
<td>Control</td>
<td>37.13</td>
<td>61.38</td>
</tr>
<tr>
<td></td>
<td>24.92</td>
<td>42.08</td>
</tr>
<tr>
<td></td>
<td>28.38</td>
<td>24.92</td>
</tr>
<tr>
<td></td>
<td>25.74</td>
<td>24.09</td>
</tr>
<tr>
<td></td>
<td>29.04</td>
<td>47.69</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>29.04 ± 1.77</td>
<td>39.96 ± 5.78</td>
</tr>
<tr>
<td>2. Phenformin (0.2mmol/kg twice daily)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>31.33 ± 1.75</td>
<td>24.59* ± 3.26</td>
</tr>
<tr>
<td>3. Wy 23675 (0.2mmol/kg twice daily)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>33.33 ± 1.75</td>
<td>22.02** ± 2.30</td>
</tr>
</tbody>
</table>
♦-D-bUU-U

J_tH.UTAT.fc AINU

YYK UVAT iU LtiM&L&

Ui^'UKF AINU

AJtfTEH

DAYS OF DKUG

if’O U K

treatmentT

'

For practical details,see text.
Statistical significance was determined by Student*s t-Test:* p<0 .0 5 ; ** p<0.01 ; *** p<0.001
Group
Treatment

1.
CMC
Control

Blood 5actate Levels
Diff.
2.
1.
Before After (2-1)
Drugs, 4 Days
Drugs,
1.02
1 .43
0 .8 7

0.96
1.02
1.17
Mean
± SEM
2.
Phenformin
(0,2mmol/kg
twice
daily).

1 .0 8

±0 .0 8
1.12
1 .2 8

0.89
0.73
0.84
1 .0 6

Mean
± SEM
3.

Wy 23675
(0 ,2 ramol/kg
twice
daily)v

1.17
1.66
1.09
0.88
1.14
1.21

+0.15
+0.23
+0.22
-0 .0 8
+0.12
+0 .04

0.11
0.19

1.19
±0.11
5 .1 3
4 .0 6
2 .2 3

3.57
2.03
1.96

.0.99 3 .1*2 ±0 .0 8 -0.53

9.27
7.53
10.88

0.10
0.10
0.09

+0.01
-0.05
+0.01
-0.01
+0,02
+0.04

+0.11
±0.05

0.11
±0.02

0.12
±0,01

+0.01
±0.01

1 0 .0 8

+4,01
+2.78
+1.34
+2.84
+1.-19
+0,90

0.13

0.14
0.17

8 .6 9
1 1 .64
1 1 .1 3
9 .1 3
1 2 .0 0
8 .8 3

+2 . 1 8

±0 .5 0

0 .9 6
1 .0 2

1.14
1.24
1.19
1.36

1.09
1.$4
1.14
1.27

+0 .0 2
+0 .2 6
-0.05
+0 .1 0
-0.05
-0.09

1.14

+0.03

Mean

1.11

ism

i0 . 0 9

La ctat e/Pvruvat e
Diff,
2.
1.
Before After (2-1)
Drugs, 4 Days
Drugs.

0.12
0.14
0.09
0.09
0.12
0.13

0 .7 6

0.94

Blood Fyruvate Levels
Diff.
2.
1.
Before After (2-1)
Drugs. 4 Days
Drugs,

io.06 ±0 ,05 .

0 .0 8

0.07

0 .1 2

0 .1 2

0.09

+0 .0 1
+0 .0 6
+0 .0 3
+0 ,0 6
+0 .0 3
-0 .0 3

4.0 .1 0
-0 .0 1

+0.13
-0 .0 1

^0.04
-0 .0 1

0 .1 3
0 .0 7

0 .1 0
0 .1 1

0.14
0 .1 1
0 .1 2

0.09
0.14
0.13

0.14

0 .1 1

+0 .0 3
+0.04
-0 .0 5
+0 .0 3
+0 .0 1
-0 ,0 3

0 .1 1
0 .0 8
0 .0 8

0 .1 1

0.14

0 .1 2

0 ,1 1

-0 .0 1

io.01

to . 01

io.02

9 .6 0

10.20
1 3 .0 0

±0.74

9.75
11.86
12.11
9.78
9.50
9.31

+0 ,48
+4.33
+1.23
+0 .1 8
-0 .7 0
-3.69

10.39
±0.51

+0 .3 1
± 1 .0 7

36.64 *•2 7 .95
1*12.24
20.27 h 9.14
25.50 +1 6 .3 7
16-.92; h 4,92
2 1 v 7 8 ; +12.95
« »***
10.24 24.17 *■13.93
±0 .6 5 i a . 7 7 * 3 . 2 2
7.23
1 0 .8 6

8.14
11.27
9.92
9.71
9.52
±0.64

2 3 .8 8

9 .6 0
9 .2 7
1 2 .1 1

9.57
8.77
11.55

+2.37
-1.59
+3.97.
-1.79
-1.15
+1.84

10.15

+0 .6 2

io.55

io.99

•i

1

i

f

250 I


SUMMARY OF METABOLITE LEVELS IN LIVER AFTER SEVEN DAYS DRUG TREATMENT.

For practical details, see text.

Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001

Metabolite levels are expressed in µmoles/gm wet liver weight - Mean ± SEM (n=6 for each group).

Abbreviations are as listed in the methods section under Measurement of Metabolites.

<table>
<thead>
<tr>
<th>Metabolite Measured</th>
<th>Control Values</th>
<th>Phenformin Values</th>
<th>% of Control</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc.</td>
<td>30.09 ± 12.49</td>
<td>21.05 ± 1.23</td>
<td>70**</td>
<td>16.25 ± 0.59</td>
</tr>
<tr>
<td>G6P</td>
<td>0.068 ± 0.0066</td>
<td>0.065 ± 0.0066</td>
<td>96</td>
<td>0.048 ± 0.005</td>
</tr>
<tr>
<td>F6P</td>
<td>0.023 ± 0.004</td>
<td>0.030 ± 0.006</td>
<td>130</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>FBP</td>
<td>0.020 ± 0.003</td>
<td>0.008 ± 0.002</td>
<td>40**</td>
<td>0.007 ± 0.003</td>
</tr>
<tr>
<td>GIAP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHAP</td>
<td>0.062 ± 0.0026</td>
<td>0.037 ± 0.004</td>
<td>60***</td>
<td>0.035 ± 0.007</td>
</tr>
<tr>
<td>3PG</td>
<td>0.315 ± 0.013</td>
<td>0.417 ± 0.031</td>
<td>132**</td>
<td>0.255 ± 0.033</td>
</tr>
<tr>
<td>2PG</td>
<td>0.023 ± 0.002</td>
<td>0.033 ± 0.006</td>
<td>144</td>
<td>0.015 ± 0.003</td>
</tr>
<tr>
<td>PEP</td>
<td>0.262 ± 0.011</td>
<td>0.787 ± 0.042</td>
<td>300***</td>
<td>0.130 ± 0.013</td>
</tr>
<tr>
<td>Lac</td>
<td>1.460 ± 0.174</td>
<td>2.533 ± 0.254</td>
<td>174**</td>
<td>1.702 ± 0.174</td>
</tr>
<tr>
<td>Pyr</td>
<td>0.165 ± 0.026</td>
<td>0.153 ± 0.031</td>
<td>93</td>
<td>0.158 ± 0.015</td>
</tr>
<tr>
<td>QAA</td>
<td>0.023 ± 0.003</td>
<td>0.018 ± 0.001</td>
<td>78</td>
<td>0.035 ± 0.006</td>
</tr>
<tr>
<td>Cit</td>
<td>0.248 ± 0.016</td>
<td>0.190 ± 0.005</td>
<td>77**</td>
<td>0.312 ± 0.022</td>
</tr>
<tr>
<td>Isocit</td>
<td>0.213 ± 0.049</td>
<td>0.132 ± 0.040</td>
<td>62</td>
<td>0.188 ± 0.014</td>
</tr>
<tr>
<td>203</td>
<td>0.037 ± 0.008</td>
<td>0.022 ± 0.005</td>
<td>60</td>
<td>0.042 ± 0.006</td>
</tr>
<tr>
<td>Glut</td>
<td>0.990 ± 0.017</td>
<td>1.220 ± 0.046</td>
<td>123***</td>
<td>0.958 ± 0.047</td>
</tr>
<tr>
<td>Mal</td>
<td>0.282 ± 0.046</td>
<td>0.412 ± 0.088</td>
<td>146</td>
<td>0.428 ± 0.034</td>
</tr>
<tr>
<td>AcCoA.</td>
<td>0.110 ± 0.011</td>
<td>0.168 ± 0.018</td>
<td>153*</td>
<td>0.090 ± 0.011</td>
</tr>
</tbody>
</table>
Table 6(iv) - cont.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control Values</th>
<th>Thenformin Values</th>
<th>% of Control</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30H-B</td>
<td>5.258 ±0.322</td>
<td>6.758 ±0.338</td>
<td>129**</td>
<td>4.917 ±0.313</td>
</tr>
<tr>
<td>Ac.Ac.</td>
<td>3.137 ±0.139</td>
<td>2.767 ±0.231</td>
<td>88*</td>
<td>2.963 ±0.173</td>
</tr>
<tr>
<td>ATP</td>
<td>1.335 ±0.048</td>
<td>1.133 ±0.052</td>
<td>85*</td>
<td>1.265 ±0.120</td>
</tr>
<tr>
<td>ADP</td>
<td>1.115 ±0.029</td>
<td>1.230 ±0.052</td>
<td>110*</td>
<td>1.053 ±0.050</td>
</tr>
<tr>
<td>AMP</td>
<td>0.273 ±0.015</td>
<td>0.343 ±0.010</td>
<td>126</td>
<td>0.255 ±0.025</td>
</tr>
<tr>
<td>Total Adenine Nucleotides</td>
<td>2.723 ±0.088</td>
<td>2.707 ±0.068</td>
<td>99</td>
<td>2.573 ±0.113</td>
</tr>
<tr>
<td>L/P</td>
<td>9.478 ±1.021</td>
<td>18.415 ±1.924</td>
<td>194**</td>
<td>10.810 ±0.502</td>
</tr>
<tr>
<td>Glut/2-OG</td>
<td>32.31 ±5.51</td>
<td>72.22 ±16.33</td>
<td>224*</td>
<td>26.80 ±5.92</td>
</tr>
<tr>
<td>3OH-B/AcAc</td>
<td>1.675 ±0.054</td>
<td>2.443 ±0.056</td>
<td>146***</td>
<td>1.653 ±0.018</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>1.197 ±0.024</td>
<td>0.923 ±0.037</td>
<td>77***</td>
<td>1.206 ±0.041</td>
</tr>
</tbody>
</table>
CROSS-OVER PLOTS OF METABOLITE LEVELS IN LIVER AFTER SEVEN DAYS DRUG

PHENFORMIN

For practical details, see text.

Wy 23675

Statistical significance was determined by Student's t-Test:

* p < 0.05 ; ** p < 0.01 ; *** p < 0.001
For practical details, see text.

Statistical significance was determined by Student's t-Test:-

- * p<0.05 ; ** p<0.01 ; *** p<0.001

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial O.D.</th>
<th>Final O.D.</th>
<th>O.D. Change</th>
<th>Minus Blank Change</th>
<th>PEPCK Activity Mean %SEM</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (no cytosol, no drug)</td>
<td>0.85</td>
<td>0.87</td>
<td>0.04</td>
<td>Mean 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (no drug)</td>
<td>0.89</td>
<td>0.38</td>
<td>0.51</td>
<td>0.46</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>0.39</td>
<td>0.51</td>
<td>0.46</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>0.37</td>
<td>0.50</td>
<td>0.45</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>0.35</td>
<td>0.52</td>
<td>0.47</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.87</td>
<td>0.36</td>
<td>0.51</td>
<td>0.46</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.88</td>
<td>0.35</td>
<td>0.53</td>
<td>0.48</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>0.43</td>
<td>0.50</td>
<td>0.45</td>
<td>2.64</td>
<td></td>
</tr>
<tr>
<td>3-mercapto-picolinate (1mM)</td>
<td>0.91</td>
<td>0.81</td>
<td>0.10</td>
<td>0.05</td>
<td>0.29</td>
<td>***</td>
</tr>
<tr>
<td>(Positive Control)</td>
<td>0.87</td>
<td>0.78</td>
<td>0.09</td>
<td>0.04</td>
<td>0.23</td>
<td>0.35±0.04</td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td>0.78</td>
<td>0.11</td>
<td>0.06</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td>0.76</td>
<td>0.13</td>
<td>0.08</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>0.79</td>
<td>0.12</td>
<td>0.07</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Wy 23675 (1mM)</td>
<td>0.83</td>
<td>0.31</td>
<td>0.52</td>
<td>0.47</td>
<td>2.76</td>
<td>2.76±0.03</td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>0.31</td>
<td>0.51</td>
<td>0.46</td>
<td>2.70</td>
<td>2.76±0.03</td>
</tr>
<tr>
<td></td>
<td>0.88</td>
<td>0.35</td>
<td>0.53</td>
<td>0.48</td>
<td>2.82</td>
<td>102%</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>0.38</td>
<td>0.52</td>
<td>0.47</td>
<td>2.76</td>
<td>2.76±0.02</td>
</tr>
<tr>
<td>Wy 23675 (0.1mM)</td>
<td>0.88</td>
<td>0.33</td>
<td>0.52</td>
<td>0.47</td>
<td>2.76</td>
<td>103%</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>0.37</td>
<td>0.53</td>
<td>0.48</td>
<td>2.82</td>
<td>2.76±0.02</td>
</tr>
<tr>
<td></td>
<td>0.91</td>
<td>0.39</td>
<td>0.52</td>
<td>0.47</td>
<td>2.76</td>
<td>2.76±0.02</td>
</tr>
</tbody>
</table>

(PEPCK activity is expressed in μmoles PEP produced/min./ml of cytosol)
Phenformin and Wy 23675 were shown to inhibit gluconeogenesis in the results reported in Chapter 5. In this chapter, the effects of these drugs have been measured on liver metabolite levels in alloxan-diabetic rats.

Alloxan-treatment resulted in a fall in body weight and a rise in blood glucose levels; both phenformin and Wy 23675-treatments lowered blood glucose levels in the diabetic rats. In the Wy 23675-treated group there was an increase in average body weight. The fall in blood glucose in the phenformin-treated group was accompanied by a rise in blood lactate levels, suggesting a possible inhibition of gluconeogenesis. Alternatively, the change in blood lactate concentration may reflect a balance between hepatic utilization and production in peripheral tissues, both of which may be affected by phenformin. Neither blood ketone bodies nor fatty acids were measured, so it is not possible to determine whether or not phenformin-treatment resulted in a diversion of lactate into the formation of these metabolites. However, total liver ketone bodies were increased only marginally (+13%) in phenformin-treated rats. On the other hand, elevations in ketone bodies have been reported to be associated with phenformin administration (24–29). Recently, Alberti et al (24) have shown that phenformin caused marked elevations in blood ketone bodies in normal and streptozotocin-diabetic rats and also that phenformin caused a dose-related inhibition of gluconeogenesis and stimulation of ketogenesis from lactate in isolated perfused rat livers; they suggested that there was an intrahepatic diversion of lactate from gluconeogenesis to ketogenesis. Such an effect could explain the rather moderate rise in lactate levels compared with the large fall in blood glucose levels shown in this work.

Wy 23675 caused a greater fall in blood glucose levels than phenformin, but had no apparent effect on blood lactate levels. This suggests that its effect in stimulating peripheral glucose utilization might be of more importance to its hypoglycaemic action than its effect of inhibiting gluconeogenesis. However, as with phenformin, no measurements
were made of blood ketone bodies or fatty acid levels and it may be that part of the hypoglycaemic action of Wy 23675 was due to inhibition of gluconeogenesis and that the lactate, that might have accumulated, was diverted to ketone body or fatty acid formation. However, total ketone body concentration in the liver was not affected by Wy 23675.

The general pattern of hepatic metabolites following seven days treatment of alloxan-diabetic rats with phenformin was similar to that reported in perfused liver by others (8,24,30).

The ATP/ADP ratio and the levels of ATP were reduced compared with the control group. This finding of a decrease in hepatic ATP levels with biguanides has been reported by many workers (31-34), who have implicated this effect in the inhibition of gluconeogenesis by these compounds. However, under certain circumstances, a reduction in the ATP/ADP ratio has been shown without an accompanying decrease in gluconeogenesis (30) and gluconeogenesis has been inhibited by biguanides without affecting the ATP/ADP ratio (30). Alberti et al (24) have also shown significant changes in gluconeogenic metabolite levels in livers exposed to phenformin without significant changes in the ATP/ADP ratio.

The results reported in this chapter also show significant increases in the ratios of lactate/pyruvate and 3-hydroxybutyrate/acetoacetate, which indicate a more reduced state in the cytoplasm and the mitochondria with phenformin-treatment.

Phenformin caused a negative cross-over in the levels of the gluconeogenic metabolites between 3-phosphoglycerate and dihydroxyacetone phosphate. Marked elevations in phosphoenolpyruvate and 3-phosphoglycerate levels and reductions in dihydroxyacetone phosphate have been similarly reported by others (8,24,30). These same workers have also shown that phenformin-treatment causes a reduction in the levels of citrate and 2-oxoglutarate: citrate was significantly lowered in the results reported in this chapter though decreases in 2-oxoglutarate levels did not reach statistical significance. Acetyl CoA levels were shown to be elevated, a finding that other workers have also reported (29,30).
has led to the suggestion (29,35) of special inhibitory effects of phenformin on phosphoglycerate kinase or on glyceraldehyde-3-phosphate dehydrogenase. However, there is no evidence to suggest a specific inhibition of an enzyme and Toews et al (29) have shown that the cross-over at this point is accompanied by too small a decrease in ATP concentration to account for inhibition of phosphoglycerate kinase. A cross-over at this point can also indicate a disturbance in the redox state resulting in a lower cytoplasmic NADH level for the glyceraldehyde-3-phosphate dehydrogenase reaction, perhaps through inhibition of the malate shuttle system. However, the lactate/pyruvate ratio was elevated with phenformin treatment, indicating a higher level of NADH in the cytoplasm — so this explanation is not likely to account for the cross-over between 3-phosphoglycerate and the triose phosphates.

Toews et al (29) reported a decrease in the conversion of pyruvate to CO₂ indicating a decreased rate of flux through the Krebs cycle. This was associated with an elevation of acetyl CoA and a decrease in citrate, isocitrate, 2-oxoglutarate, glutamate and malate. They suggested an inhibition of the Krebs cycle between acetyl CoA and citrate. Alberti et al (24) have shown that phenformin caused an accumulation of three-carbon metabolites and decreased concentrations of citrate and 2-oxoglutarate; similar results are reported in this work.

Haekel and Haekel (30) have suggested that pyruvate carboxylation could be specifically inhibited by phenformin and this could account for the increase in acetyl CoA and the decrease in oxaloacetate and, consequently, the decrease in citrate and 2-oxoglutarate. However, they reported that phenformin in concentrations up to 0.8 mM had no effects on pyruvate carboxylase activity in vitro.

Alberti et al (24) have suggested that a change in the malate/oxaloacetate ratio, reflecting a more reduced state in the cell, could also explain the fall in citrate and 2-oxoglutarate. The results reported in this chapter show a rise in malate and a fall in oxaloacetate levels, neither of which were significant. Alberti et al (24) showed a
significant elevation of malate levels in perfused livers with 1mM phenformin, but this was not significant with 2mM phenformin; they did not measure oxaloacetate levels. They suggested that because of the shortage of acetyl CoA acceptor (i.e., oxaloacetate) for citrate synthesis, the acetyl CoA is diverted into ketone body synthesis and this explains the enhanced ketogenesis seen with biguanides. The results reported in this chapter lend some support to the mode of action suggested by Alberti et al (24), although the change in the oxaloacetate levels was not significant.

Measurements of gluconeogenesis from different substrates (Chapter 5) suggested that phenformin might inhibit gluconeogenesis at a step in the pathway between pyruvate and fructose-6-phosphate. Although the measurement of metabolite levels has provided some evidence for an effect of phenformin at the glyceraldehyde-3-phosphate dehydrogenase step, there was no evidence to suggest an inhibition of gluconeogenesis in the conversion of fructose bisphosphate to fructose-6-phosphate.

The pattern of hepatic metabolites following seven days treatment with Wy 23675 differed markedly from that seen with phenformin treatment. There was a negative cross-over between malate and phosphoenolpyruvate with a reduction in the level of all metabolites from phosphoenolpyruvate to glucose. Neither lactate, pyruvate nor oxaloacetate concentration were significantly different from the controls, although oxaloacetate was elevated by 50% (the extreme lability of oxaloacetate makes precise measurement of this metabolite very difficult).

The cross-over between malate and phosphoenolpyruvate suggests that Wy 23675 is inhibiting the enzyme phosphoenolpyruvate carboxykinase (PEPCK). 3-mercaptopicolinate is a known inhibitor of this enzyme (13, 14, 36, 37) and cross-over plots obtained by other workers with this drug (13, 14) look similar to that reported here for Wy 23675. The present work and that of Goodman (14) do not show an elevation of lactate levels as a consequence of inhibition of PEPCK as has been shown with 3-mercaptopicolinate by other workers (13). The finding that Wy 23675
a very marked effect on gluconeogenesis when administered in vivo. The rise in malate after Wy 23675-treatment was small (152%) compared with results for 3-mercaptopicolinate reported by other workers (ie: 908% (13)) and this would support the argument that Wy 23675 is not a very effective inhibitor of PEPCK.

There was a significant elevation in the levels of citrate following Wy 23675-treatment: this was probably a consequence of the inhibition of PEPCK resulting in a greater availability of oxaloacetate for citrate as well as malate synthesis.

There was no evidence from the metabolites measured in this in vivo study to suggest that Wy 23675 inhibited gluconeogenesis at the step controlled by fructose bisphosphatase and phosphofructokinase (cf. Chapter 5).

The possibility that Wy 23675 could inhibit PEPCK was investigated by measurement of the activity of this enzyme in vitro. However, the present experiment failed to show any direct effect of Wy 23675 on PEPCK, while a clear inhibition was shown with 3-mercaptopicolinate. There are several possibilities which could explain this negative result.

Liver metabolite levels were measured in freeze-clamped livers following seven days, twice daily, treatment in vivo with Wy 23675; and it may be that chronic exposure of the liver to Wy 23675 is necessary in order to see an effect on PEPCK.

There is also the possibility that it is a metabolite of Wy 23675, and not Wy 23675 itself, that is having an inhibitory effect on PEPCK. As it is not a whole-cell preparation that is used for the assay of PEPCK such a metabolite might not be formed in the cytosol — particularly in the short incubation period used.

However, it is also quite possible that Wy 23675 has no effect on PEPCK. The inhibition of the transport of metabolites in the gluconeogenic pathway across the mitochondrial membrane can also inhibit gluconeogenesis.
as has been shown with diethylmalonate, which inhibits pyruvate transport, and n-butylmalonate, which inhibits malate transport (43). The reason these compounds are effective inhibitors of gluconeogenesis is because pyruvate carboxylase is located within the mitochondrion, while PEPCK is located in the cytosol, so that inhibition of pyruvate transport prevents pyruvate from reaching pyruvate carboxylase and inhibition of malate transport prevents oxaloacetate from leaving the mitochondrion to reach PEPCK in the cytosol (oxaloacetate cannot readily cross the mitochondrial membrane and, under reduced conditions, is transported into the cytoplasm in the form of malate). Inhibition of malate transport could be expected to give a similar picture with gluconeogenic metabolite levels to that seen with Wy 23675 - namely, an increase in malate and a decrease in PEP, giving the appearance of an inhibition of PEPCK. This could provide an alternative explanation for the mechanism of Wy 23675-inhibition of gluconeogenesis.
References.


Overall Discussion.
OVERALL DISCUSSION.

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OVERALL DISCUSSION.

Wy 23675 is a novel hypoglycaemic agent, which has been shown to have a number of different actions, each of which could contribute to its ability to lower blood glucose levels in vivo. These actions include the stimulation of insulin release, an insulin-independent stimulation of glucose utilization, and an insulin-independent inhibition of hepatic gluconeogenesis.

The Relative Importance Of The Insulin-Independent Effects Of Wy 23675 On Glucose Utilization And Gluconeogenesis.

Wy 23675 has been shown to lower blood glucose levels to a large degree in both normal and alloxan-diabetic rats (see Table 7(i)), so it appears that the insulin-independent action of Wy 23675 is significant in relation to its overall hypoglycaemic activity. Most of the work described in this thesis concerns the insulin-independent hypoglycaemic actions of Wy 23675.

Wy 23675 has been shown to have insulin-independent effects both on glucose utilization and on glucose production and to lower blood glucose levels in both normal, fed and fasted rats. However, it is not possible to determine the relative contributions of the insulin-independent effects of Wy 23675 to the lowering of blood glucose levels following oral administration in vivo, as insulin release is stimulated under these circumstances. It is also difficult to distinguish between the relative contributions made by inhibition of glucose production and stimulation of glucose utilization to the hypoglycaemic action of the drug in the alloxan-diabetic rat, because of the exaggerated importance of gluconeogenesis even in the fed animal.

The results in Chapter 2 showed that Wy 23675 caused an increase in ($^3$H-2)-glucose uptake from the blood stream and simultaneous measurements showed that the decrease in blood glucose levels occurred without a change in plasma insulin levels in certain animal models. However,
due to the non-steady state conditions, it was not possible to calculate the proportion of the fall in blood glucose that was due to increased glucose utilization, rather than to inhibition of gluconeogenesis.

Some information on this matter may be gained from a comparison of the concentration of Wy 23675 required to affect glucose uptake by hemidiaphragm and adipocytes and that required to affect gluconeogenesis by hepatocytes in vitro [see Table 7(ii)]. In tissues from the normal, fed rat, glucose utilization was significantly increased by Wy 23675 in concentrations in the region of 20-200μM and similar effects were seen with tissues from alloxan-diabetic rats. In contrast, the inhibitory effects of Wy 23675 on basal rates of gluconeogenesis were not significant in hepatocytes from normal rats until a drug concentration of 20mM was obtained—some 100-1000 fold higher than the concentration required for effects on glucose uptake. This contrasts markedly with glucagon-stimulated gluconeogenesis where Wy 23675 caused significant inhibition at 2μM: this latter effect could provide an explanation for the more marked hypoglycaemic effect of Wy 23675 in the normal, starved rat where gluconeogenesis is enhanced as compared with the normal, fed rat. However, in hepatocytes from alloxan-diabetic rats, Wy 23675 had significant inhibitory effects on gluconeogenesis at 20μM—a concentration lower than that required for significant effects on glucose utilization in tissues from alloxan-diabetic rats.

If these in vitro findings can be applied to the in vivo situation (where an estimated in vivo drug concentration following an oral hypoglycaemic dose is 100μM), then they suggest that, in the normal fed rat, Wy 23675 owes its insulin-independent hypoglycaemic activity primarily to stimulation of glucose utilization with little, if any, part being played by inhibition of gluconeogenesis. In the alloxan-diabetic rat, inhibition of gluconeogenesis would appear to contribute to a much greater extent to the hypoglycaemic activity of Wy 23675 along with the increase in glucose utilization. However, the maximal inhibition of
gluconeogenesis is approximately 50-60%, while the maximal increase in glucose utilization is in the region of 100-120%, so that in the alloxan-diabetic rat, as well as in the normal rat, a major part of the hypoglycaemic activity of Wy 23675 may well be attributable to stimulation of glucose utilization.

Wy 23675 has been compared in this thesis with the sulphonylurea, tolbutamide, and the biguanide, phenformin. It is clear from the results obtained that, as anticipated, tolbutamide is acting by stimulation of insulin release with a consequent increase in glucose utilization and decrease in elevated rates of gluconeogenesis.

The Relative Importance Of The Effects Of Phenformin On Glucose Utilization And On Gluconeogenesis.

Phenformin has been shown to stimulate glucose utilization and to decrease gluconeogenesis independent of insulin release. It has also been shown to lower blood glucose levels in fasted, normal rats, but to have no effect in fed, normal rats (see Table 7(iii)). The major difference between these two situations is the elevated rate of gluconeogenesis in the fasted rat, which suggests that inhibition of gluconeogenesis by phenformin is the mechanism by which it lowers blood glucose levels under these circumstances. Phenformin also lowered blood glucose levels in the glucose-loaded and alloxan-diabetic rats (Table 7(iii)). The glucose-loaded rat has elevated blood glucose levels and reduced rates of gluconeogenesis, which suggests that phenformin has significant effects on glucose utilization in situations where blood glucose levels are elevated. The ability of phenformin to lower blood glucose levels in the alloxan-diabetic rat may thus be a combination of stimulation of glucose utilization and inhibition of gluconeogenesis.

A comparison of the concentration of phenformin required to produce different effects in vitro may shed further light on the relative importance of glucose utilization and gluconeogenesis in the normal and alloxan-diabetic rat (see Table 7(iv)). There were no significant
effects on basal rates of either gluconeogenesis or glucose utilization in isolated tissues from normal rats until concentrations of phenformin in the region of 0.2-2mM were attained; these concentrations are well above those obtained with therapeutic administration of the drug [1-10µM (1)]. However, phenformin significantly inhibited gluconeogenesis that was stimulated by glucagon in hepatocytes from normal rats in concentrations as low as 2µM - this effect could explain its significant hypoglycaemic activity in the fasted rat, while being inactive in the fed animal. A similar effect was seen with Wy 23675 and suggests that enhanced rates of gluconeogenesis are far more sensitive to the actions of these drugs than basal rates.

Mechanism Of Action Of Phenformin And Wy 23675 On Glucose Utilization

For the reasons already discussed, it is proposed that a major part of the hypoglycaemic action of Wy 23675 is due to its effects on glucose utilization. This differs from the findings for phenformin, which suggest that inhibition of enhanced rates of gluconeogenesis may be of greater importance, although effects of phenformin on glucose utilization were observed in the alloxan-diabetic rat.

The mechanisms by which these drugs exert their effects on glucose utilization remain unclear. The possibility that biguanides reduce the negative feedback of glucose-6-phosphate on hexokinase, which occurs in diabetes due to excessive oxidation of fatty acids by muscle, has been proposed by Frayn and Adnitt (2). They have suggested that biguanides increase the rate of removal of glucose-6-phosphate by increasing glycogen synthesis. However, the results reported in this thesis show a decrease in glycogen synthesis.

There is a reduction in the membrane transport of glucose in muscles from diabetic animals (3-6), so it is possible that biguanides could increase glucose utilization by increasing glucose transport. However, this seems very unlikely as it well established that biguanides inhibit glucose transport across the intestine (7-10).
Glycolytic flux is believed to be controlled primarily at the level of phosphofructokinase (11) and this enzyme is activated by a decreased level of ATP and increased levels of fructose bisphosphate and AMP (12). Biguanides have been shown to inhibit mitochondrial respiration and oxidative phosphorylation (13, 14, for further references, see Introduction), resulting in a reduction in ATP and an increase in AMP (see also results reported in Chapter 6). Thus, biguanides could increase glycolytic flux (and, consequently, glucose utilization) by changing the ATP/AMP ratio.

Wy 23675 has been shown to increase glucose utilization by muscle without a subsequent increase in lactate output (Chapter 3) and also to increase glucose incorporation into adipocyte lipids (Chapter 4). It has been proposed that activation of pyruvate dehydrogenase could account for these observations. However, there was no evidence that Wy 23675 activated pyruvate dehydrogenase in the liver (see Chapter 5, discussion), although there was evidence for an inhibition of gluconeogenesis without a subsequent elevation in blood lactate levels (see Chapter 6). No direct measurements of the effect of Wy 23675 on pyruvate dehydrogenase have been made, so it is not possible to eliminate activation of this enzyme as a mechanism involved in the stimulation of glucose utilization.

It is possible Wy 23675 stimulates glucose utilization by increasing glucose transport into the cell, but this could not explain the apparent increase in aerobic glycolysis unless pyruvate dehydrogenase was also activated.

**Mechanisms Of Action Of Phenformin And Wy 23675 On Gluconeogenesis.**

Both phenformin and Wy 23675 have also been shown to inhibit gluconeogenesis (see Chapter 5); and to a greater extent in the liver of alloxan-diabetic rats than in normal rats. Studies of liver metabolite levels following in vivo treatment with phenformin and Wy 23675 (see Chapter 6) provided some evidence for their mechanisms of action.

Phenformin treatment resulted in a reduction in the ATP/ADP ratio and a more reduced state in the cell, suggesting that it was inhibiting
gluconeogenesis, under the conditions employed in this study, by inhibiting mitochondrial respiration and oxidative phosphorylation. Other workers (15-17) have shown effects of phenformin on gluconeogenesis independent of any effect on oxidative phosphorylation: Schafer (18) and Davidoff (19) have proposed mechanisms to explain such an inhibition by the biguanides (these theories are discussed in the Introduction to this thesis).

Wy 23675 has been shown to inhibit gluconeogenesis independent of any effects on oxidative phosphorylation (see Chapter 6) and a proposed mechanism of action is by inhibition of the enzyme phosphoenolpyruvate carboxykinase. However, direct inhibition of the enzyme in vitro by the drug was not demonstrated (see Chapter 6).

Summary.

In summary, Wy 23675 has a hypoglycaemic action which contains an insulin-independent component. This component involves both stimulation of glucose utilization and inhibition of gluconeogenesis. It is proposed that the effects of Wy 23675 on glucose utilization make up the most significant part of its insulin-independent hypoglycaemic action and that this effect may be mediated by activation of pyruvate dehydrogenase. It is also proposed that Wy 23675 inhibits gluconeogenesis at a point in the pathway between malate and phosphoenolpyruvate, possibly by inhibiting the enzyme phosphoenolpyruvate carboxykinase. Further work is required to elucidate the mechanisms involved in these actions.
<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>7(i)</td>
<td>Effects of Wy 23675 on Blood Glucose Levels in 24-hr Fasted, Fed, Glucose-Loaded and Alloxan-Diabetic Rats.</td>
</tr>
<tr>
<td>7(ii)</td>
<td>Comparison of the Concentrations of Wy 23675 Required to Produce &quot;In Vitro&quot; Effects on Glucose Metabolism</td>
</tr>
<tr>
<td>7(iii)</td>
<td>Effects of Phenformin on Blood Glucose Levels in 24-hr Fasted, Fed, Glucose-Loaded and Alloxan-Diabetic Rats.</td>
</tr>
<tr>
<td>7(iv)</td>
<td>Comparison of the Concentration of Phenformin Required to Produce &quot;In Vitro&quot; Effects on Glucose Metabolism.</td>
</tr>
</tbody>
</table>
Table 7(i).

EFFECTS OF Wy 23675 ON BLOOD GLUCOSE LEVELS IN 24-HR FASTED, FED, GLUCOSE-LOADED AND ALLOXAN-DIABETIC RATS.

For details of methods and data, see Table 1, Chapter 1.

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>% Change in Blood Glucose Levels Due to Wy 23675 (0.2mmol/kg p.o.) Relative to Control at the Following Times:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>24-hr Fasted Rats</td>
<td>-51%***</td>
</tr>
<tr>
<td>Fed Rats</td>
<td>-27%***</td>
</tr>
<tr>
<td>Glucose-Loaded Rats</td>
<td>-39%**</td>
</tr>
<tr>
<td>Alloxan-Diabetic Rats</td>
<td>-50%*</td>
</tr>
</tbody>
</table>

Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
Table 7(ii)

Comparison of the concentrations of Wy 23675 required to produce 'in vitro' effects on glucose metabolism.

For details of methods and data, see Chapters 3, 4 and 5.

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>Wy 23675 Concentration</th>
<th>% Change in Glucose Utilization by:</th>
<th>% Change in Gluconeogenesis from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hemi-Diaphragm</td>
<td>Adipocyte</td>
</tr>
<tr>
<td>Normal</td>
<td>2μM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rat</td>
<td>20μM</td>
<td>+13%</td>
<td>+37%***</td>
</tr>
<tr>
<td></td>
<td>200μM</td>
<td>+40%*</td>
<td>+172%***</td>
</tr>
<tr>
<td></td>
<td>2mM</td>
<td>+97%***</td>
<td>+206%***</td>
</tr>
<tr>
<td></td>
<td>20mM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alloxan-Diabetic</td>
<td>2μM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rat</td>
<td>20μM</td>
<td>+21%</td>
<td>+28%</td>
</tr>
<tr>
<td></td>
<td>200μM</td>
<td>+85%**</td>
<td>+94%***</td>
</tr>
<tr>
<td></td>
<td>2mM</td>
<td>+114%**</td>
<td>+124%***</td>
</tr>
</tbody>
</table>

where I/P = 10mM lactate plus 1mM pyruvate

I/P/G = 10mM lactate plus 1mM pyruvate stimulated by 50nM glucagon

Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
**Table 7(iii).**

**EFFECTS OF PHENFORMIN ON BLOOD GLUCOSE LEVELS IN 24-HR FASTED, FED, GLUCOSE-LOADED AND ALLOXAN-DIABETIC RATS.**

For details of methods and data, see Table 1, Chapter 1.

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>% Change in Blood Glucose Levels Due to Phenformin (0.2mmol/kg p.o.) Relative to Control at the Following Times:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>24-hr Fasted Rats</td>
<td>-27%**</td>
</tr>
<tr>
<td>Fed Rats</td>
<td>-4%</td>
</tr>
<tr>
<td>Glucose-Loaded Rats</td>
<td>-45%</td>
</tr>
<tr>
<td>Alloxan-Diabetic Rats</td>
<td>-28%*</td>
</tr>
</tbody>
</table>

Statistical significance was determined by Student's t-Test:

* p<0.05 ; ** p<0.01 ; *** p<0.001
**Table 7(iv).**

**COMPARISON OF THE CONCENTRATIONS OF PHENFORMIN REQUIRED TO PRODUCE 'IN VITRO' EFFECTS ON GLUCOSE METABOLISM.**

For details of methods and data, see Chapters 3, 4 and 5.

<table>
<thead>
<tr>
<th>Animal Treatment</th>
<th>Phenformin Concentration</th>
<th>% Change in Glucose Utilization by:</th>
<th>% Change in Gluconeogenesis from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hemi-Diaphragm</td>
<td>Adipocyte</td>
</tr>
<tr>
<td>Normal</td>
<td>2 μM</td>
<td>-10%</td>
<td>-</td>
</tr>
<tr>
<td>Rat</td>
<td>20 μM</td>
<td>+2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>200 μM</td>
<td>+35%**</td>
<td>+8%</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>-</td>
<td>+67%***</td>
</tr>
<tr>
<td></td>
<td>20 mM</td>
<td>-</td>
<td>+114%***</td>
</tr>
<tr>
<td>Alloxan-</td>
<td>2 μM</td>
<td>+21%</td>
<td>-</td>
</tr>
<tr>
<td>Diabetic</td>
<td>20 μM</td>
<td>+53%*</td>
<td>+23%</td>
</tr>
<tr>
<td>Rat</td>
<td>200 μM</td>
<td>+73%**</td>
<td>-4%</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>-</td>
<td>+69%*</td>
</tr>
</tbody>
</table>

where $L/P = 10$ mM lactate plus $1$ mM pyruvate

$L/P/G = 10$ mM lactate plus $1$ mM pyruvate stimulated by $50$ nM glucagon

Statistical significance was determined by Student's $t$-Test:

- $* p<0.05$ ; $** p<0.01$ ; $*** p<0.001$
Overall Discussion References.