ABSTRACT

Carrier-free $^{65}\text{Zn}$ and $^{67}\text{Ga}$ produced in the M.R.C. cyclotron were used to study the metabolism of zinc and gallium in rats. The appropriate literature has been reviewed.

Zinc excretion has been studied using a sub-physiological tracer dose of $^{65}\text{Zn}$. In 25 days 80% of the dose was excreted in the faeces. The secretions of the pancreas and liver contributed approx. 30% of the faecal $^{65}\text{Zn}$, and it is suggested that the origin of approx. 70% of faecal zinc is the secretions of the Paneth cells and goblet cells and other gastro-intestinal secretions.

Evidence is presented that the zinc secreted in bile is in the form of a macromolecular complex.

The distribution and turnover of zinc in a number of soft tissues have been studied.

The effect of oral $\text{ZnSO}_4$ on the tissue retention of endogenous $^{65}\text{Zn}$ has been studied.

The significance of these results and their relevance to the use of radionuclides of zinc as pancreas and prostate gland scanning agents have been discussed.

Tissue distribution and excretion of $^{67}\text{Ga}$ and its localisation in transplanted tumours have been studied. Gallium was retained in the body due to plasma protein binding and tissue deposition, resulting in its slow excretion. $^{67}\text{Ga}$ localised mainly in viable tumour cells.
ACKNOWLEDGEMENTS

Professor D.V. Parke and Dr. D.K. Bewley are gratefully thanked for providing me with the opportunity to carry out this course of study and for their guidance.

My sincere thanks are due to Dr. L.J. King who, as my supervisor, guided me throughout the entire course of this work and without whom some aspects of this work would not have been possible.

I wish to express my sincere gratitude to Mr. D.D. Vonberg, the director of the Cyclotron Unit for the facilities to carry out this work. I should like to thank the Medical Research Council for providing financial support.
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The abbreviations and symbols used in the text comply with the rules laid down by the Biochemical Journal in the booklet "Instructions to Authors" (1973).
CHAPTER I

GENERAL INTRODUCTION

During the last two decades there has been an unprecedented expansion of basic research in nuclear science, accompanied by a vast programme of research and development in the field of nuclear medicine. The tangible effects have been a remarkable increase in the kind and the number of radioactive pharmaceuticals available for clinical diagnostic and therapeutic use (Windeyer, 1972).

1-1 Tumour scanning agents

Great interest has been shown in the development of compounds for selective concentration in tumours which would be of considerable diagnostic value. An ideal diagnostic agent must be able to establish:

(a) The presence of tumours,
(b) The location of the primary tumour in patients with proven cancer,
(c) The metastatic spread of the primary tumour and location of secondary lesions.

The presence of the diagnostic agent in the abnormal area must be detectable with relative ease and rapidity. Such ideal tumour diagnostic agents are non-existent and those that fulfil some of the qualities of the hypothetical
agent are rare.

Radionuclides with penetrating radiation fulfil the requirement of ease of detectability. A radionuclide can indicate the presence of a tumour if it concentrates within or extraneous to the tumour in relatively high proportions. Concentration within the tumour in excess of that of surrounding tissue shows a "hot-lesion" and the reverse phenomenon shows a "cold-lesion". In order to be of value in diagnostic applications, a radionuclide should have a long physical half-life compared with the duration of the investigation in which it is to be used. However, the biological half-life should be sufficiently short for it not to constitute a radiation hazard to the recipient. New radionuclides which comply with these requirements, and also emanate useful radiation required by detection instruments are being produced. In the selection of metal nuclides, emphasis at the present time has principally been placed on the physical characteristics, such as half-life, the principal emissions and their energies, the nuclear reactions involved in their production and their efficiency of detection. The biochemical, physiological and pharmacological considerations for the selection and use of these radionuclides have been subordinate. Occasionally, some radio-nuclides have been selected on biologically rational grounds. The success of radioiodine for thyroid physiology (Hertz, 1938) nurtured hopes that other elements would be specific for certain organs or tumours; nuclides of zinc were proposed for the diagnosis of carcinoma of the pancreas (Meschan et al.,
1959), and the prostate (Johnston et al., 1968) both organs with a high affinity for zinc. However, the success obtained with the artificial element, $^{99m}$Tc in scanning for tumours of the brain, the thyroid, the liver and lung (Silver, 1968) justifies the investigation of any nuclide for initial test screens to assess its ability to concentrate in tumours. Therefore these elements need not necessarily be nuclides of endogenous elements, and can be analogues of physiological substances, anutrients or antimetabolites.

Currently, the radionuclides $^{62}$Zn and $^{67}$Ga are produced in the M.R.C. cyclotron, and these are screened in order to evaluate their diagnostic value in tumour localisation. The availability of these elements in carrier-free form permits the study of their metabolic and pharmacokinetic properties at sub-physiological levels, which is the subject reported in this thesis.

1-2 Zinc

1-2-1 Introduction

Zinc is unequivocally required in trace amounts for the growth and development of plants and animals, and is found ubiquitously distributed in the vertebrate body (Vallee, 1962). Zinc has been shown to be associated with several enzymes (O'Dell & Campbell, 1970). Dietary intake of zinc below the normal requirement causes disease and the condition can be reversed by dietary supplementation or by oral zinc therapy (Prasad, 1966).
The absorption, tissue distribution and the metabolism of zinc have been documented for some eutherian mammals of the domestic and laboratory environment (Table 1-1).

Table 1-1 Zinc metabolism studies in mammals

<table>
<thead>
<tr>
<th>Order</th>
<th>Animals</th>
<th>Study</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodentia</td>
<td>Mouse</td>
<td>Sheline et al.</td>
<td>(1943)</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Gilbert &amp; Taylor</td>
<td>(1956)</td>
</tr>
<tr>
<td>Lagomorpha</td>
<td>Rabbit</td>
<td>Cotzias et al.</td>
<td>(1961)</td>
</tr>
<tr>
<td>Carnivora</td>
<td>Dog</td>
<td>Sheline et al.</td>
<td>(1943)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Robertson &amp; Burns</td>
<td>(1963)</td>
</tr>
<tr>
<td>Artiodactyla</td>
<td>Cattle</td>
<td>Miller</td>
<td>(1963)</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>McKenney et al.</td>
<td>(1962)</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>Hoekstra et al.</td>
<td>(1956)</td>
</tr>
<tr>
<td>Primates</td>
<td>Monkey</td>
<td>Underwood</td>
<td>(1971)</td>
</tr>
<tr>
<td></td>
<td>Man</td>
<td>Spencer et al.</td>
<td>(1965)</td>
</tr>
</tbody>
</table>

1-2-2 Absorption

Since the initial study of Stern et al. (1935) on the zinc requirement of the rat for growth, many studies have been reported on zinc absorption.

In an experiment in guinea-pigs, Skog et al. (1964)
found that percutaneous $^{65}\text{ZnCl}_2$ was very slowly absorbed, less than 1% of the dose being absorbed over 5 h. Subcutaneously deposited $^{65}\text{ZnCl}_2$ in rats resulted in approx. 92% absorption in 24 h (Khristov, 1970). Absorption of $^{65}\text{Zn}$ from the peritoneum of rats occurred almost completely in the first 24 h following injection (Feaster et al., 19565)

Ballou and Thompson (1961) showed that 25% of a dose of $^{65}\text{ZnCl}_2$ deposited in the stomach of rats was absorbed from the stomach within 24 h. Khristov (1970) stated that other soluble compounds of zinc such as zinc acetate, zinc citrate and zinc glycine complex are rapidly removed from the bloodstream and readily absorbed from sites other than the surface of the skin.

Sparingly soluble salts of zinc, such as the carbonate and the phosphate are only slowly absorbed from subcutaneous and intramuscular sites. These compounds and zinc dithizone complex are retained in the lungs of rats following intravenous injection (Banks et al., 1950). Phytate, a natural chelating agent present in plants, is known to reduce the absorption of dietary zinc in animals (O'Dell & Savage, 1960) and man (Reinhold et al., 1973).

Dietary zinc is absorbed mainly in the distal portion of the small intestine. Campen and Mitchell (1965) showed that in the ligated portions of the small intestine of the rat, $^{65}\text{ZnCl}_2$ is absorbed in the duodenal, ileal and the jejunal portions to an extent of 5.41%, 2.04% and 0.66% of the dose respectively, in 3 h. $^{65}\text{ZnCl}_2$ uptake in the everted duodenal sacs was not affected by $\text{CN}^-$ or the absence of
oxygen (Oberleas et al., 1966). Non-radioactive zinc added to drinking water of rats, containing $^{65}$ZnCl$_2$ effectively reduced $^{65}$Zn absorption (Avrunina, 1970). By the addition of 0.04 mg zinc and 2.0 mg zinc as non-radioactive ZnSO$_4$ to the daily rations which also contained 1.0 µCi $^{65}$Zn, absorption of $^{65}$Zn was 5.5% and 2.9% compared with an absorption of 6.4% for the control animals. Cotzias et al. (1962) demonstrated that zinc but not Cu, Ga, Mn or Cd, when used as a dietary or intraperitoneal metabolic load, lowered absorption and enhanced excretion of $^{65}$Zn in mice. With an increment of ZnSO$_4$ added to the diet from 50 to 500 p.p.m., absorption of intraperitoneally administered $^{65}$ZnCl$_2$ decreased from 34% to 16%. Ballou and Thompson (1961) proposed that zinc absorption in the gastro-intestinal tract is age-dependent. The amount of $^{65}$Zn retained in the body, 24 h after an oral dose, decreased from 90% to 15% in the 6 and 26 day old Sprague-Dawley rats respectively. Suso and Edwards (1968) observed recognisable differences in $^{65}$Zn absorption in chicken and attributed this to heritable factors.

Duodenal uptake of zinc has been shown to be facilitated by the amino acids, glycine and methionine (O'Dell, 1969). A copper binding protein of approx. mol.wt.10,000, in the duodenal mucosal cell supernatant of the chick was discovered by Starcher (1969). Zinc and cadmium have also been shown to associate with this protein. Starcher postulated that copper, zinc and cadmium are absorbed in the gut by the same mechanism, on the basis of the cytoplasmic protein binding. However, there is no evidence to associate cytoplasmic protein
Table 1-2  Endogenous zinc content in some selected tissues in the experimental rats and man

<table>
<thead>
<tr>
<th></th>
<th>Collip rat</th>
<th>August rat</th>
<th>Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body zinc content (µg)</td>
<td>2950 ± 300*</td>
<td>1.4-2.3 x 10^6**</td>
<td></td>
</tr>
<tr>
<td>Soft tissue content (µg/g)</td>
<td>15 - 30</td>
<td>15 - 30</td>
<td>20 - 30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Tissue</th>
<th>µg zinc / g wet wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Liver</td>
<td>30 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Pancreas</td>
<td>23 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>23 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>19 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>24 ± 3.4</td>
</tr>
<tr>
<td>B</td>
<td>Bone</td>
<td>233 ± 22</td>
</tr>
<tr>
<td></td>
<td>Voluntary muscle</td>
<td>13 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>180 ± 45</td>
</tr>
<tr>
<td>C</td>
<td>Study</td>
<td>(1)</td>
</tr>
</tbody>
</table>

* µg/100 g body weight  
** µg/70 kg body weight  
(1) Mawson & Fisher (1951)  
(2) Gilbert & Taylor (1956)  
(3) Siegel et al. (1961)
binding with duodenal absorption. Zinc interaction with cytoplasmic protein may merely facilitate transport processes at the cell membrane. Recently, Campen and Kowalski (1971) have reported the presence of a similar zinc binding protein in the duodenal cells of the rat. Thus, zinc absorption from the gut does not appear to involve an active process, nevertheless, the foregoing evidence indicates that it is highly selective.

1-2-3 Tissue distribution of endogenous zinc

The distribution of endogenous zinc in two strains of adult laboratory rat and man are shown in Table 1-2. In the body the highest zinc content is found in the skeleton, with lower levels in the muscle and liver, the zinc content being, approx. 26.7%, 17.2% and 4.1%, respectively, of the total body content, in the rat. In humans, in comparison with the rat, the zinc content is higher in the tissues studied. This is illustrated by tissues in groups A and B (Table 1-2) and the prostate, the group A tissues showing the highest difference. But both species contain about 30 μg zinc per gram body weight. In rats the zinc content of the skeleton has been found to increase with age (Taylor, 1961):

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Zinc content in bone (μg zinc/ g wet wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>77 ± 7</td>
</tr>
<tr>
<td>103</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>313</td>
<td>133 ± 8</td>
</tr>
<tr>
<td>679</td>
<td>191 ± 5</td>
</tr>
</tbody>
</table>
Tissue distribution of $^{65}$Zn

$^{65}$Zn (ZnCl$_2$) has been used extensively to investigate tissue distribution of this element, with the assumption that the tracer seeks the normal compositional pattern of the stable element in the body. $^{65}$Zn has been assayed by scintillation counting of the gamma radiation present in the dissected organs and tissues, withdrawn from the body at different time intervals after administration. The findings of Muller (1946), Gunn et al. (1955), Khristov (1970) and others have resulted in the following classification of tissues according to their exchange rates for intravenous $^{65}$ZnCl$_2$.

**Group A Tissues**

Highest $^{65}$Zn concentration between 0 to 2 h, with subsequent rapid loss:

(1) Pancreas
(2) Liver
(3) Kidney
(4) Duodenum

**Group B Tissues**

Intermediate $^{65}$Zn concentration with subsequent rapid loss:

(1) Spleen
(2) Lung
(3) Stomach
(4) Large intestine
(5) Heart muscle
Group C Tissues

Initial low $^{65}$Zn concentration with subsequent increase over a long period (days):

(1) Skeleton
(2) Skin and hair
(3) Testes
(4) Prostate gland
(5) Voluntary muscle
(6) Erythrocytes

In the mouse, the rat and the dog the pattern of tissue distribution of $^{65}$Zn is similar. The peak concentrations of $^{65}$Zn in group A and B tissues are reached around 8 h in the dog (Meschan et al., 1959), around 2 h in the rat (Stand et al., 1962), and earlier than 2 h in the mouse (Sheline et al., 1943) after i.v. $^{65}$Zn.

Initially, the tissue distribution of $^{65}$Zn does not follow the normal compositional pattern of endogenous zinc. In the rat, skeleton and the skeletal muscle contain approx. 26.7% and 17.2% of the whole body zinc content, but the liver with approx. 4.1% zinc content shows an initial high concentration of $^{65}$Zn (Gilbert & Taylor, 1956). At 24 h the liver contained 15.7%, the skeleton, 20.7% and the skeletal muscle, 14.2% of an intravenous dose of $^{65}$Zn. The rapid release of $^{65}$Zn from the liver, its slow release from the muscle and its retention in the bone can be seen in Table 1-3. Mouse (Sheline et al., 1943), rat (Gilbert & Taylor, 1956), dog (Meschan et al., 1959), and man (Spencer et al., 1965) have been shown to concentrate maximally, about 25%, 35%, 38%, and 64% respectively, of
a trace dose of intravenous $^{65}\text{Zn}$ in the liver.

The initial high concentration of $^{65}\text{Zn}$ in the liver cannot be explained by the $^{65}\text{Zn}$ in the blood alone since a similarly highly vascular tissue, the spleen, contained less $^{65}\text{Zn}$ per gram of tissue at all times (Table 1-4). The high concentration in the liver and kidneys is therefore probably due to $^{65}\text{Zn}$ incorporation into intracellular components such as the cellular macromolecules.

Table 1-3 $^{65}\text{Zn}$ turnover in the liver, skeleton and the voluntary muscle of the rat

<table>
<thead>
<tr>
<th>Endogenous zinc</th>
<th>Distribution of intravenous $^{65}\text{Zn}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculation as:</td>
<td></td>
</tr>
<tr>
<td>Percentage of</td>
<td>Percentage of initial dose</td>
</tr>
<tr>
<td>total body zinc</td>
<td>Time after injection (days)</td>
</tr>
<tr>
<td></td>
<td>0.04 1 4 7 35 100</td>
</tr>
<tr>
<td>Liver 4.1*</td>
<td>25    15.7 6.9 4.5 1.6* 0.4*</td>
</tr>
<tr>
<td>Skeleton 24.7*</td>
<td>-     20.7 15.0 16.4 11.3* 14.5*</td>
</tr>
<tr>
<td>Muscle 17.2*</td>
<td>-     14.2 16.9 15.5 9.5* 2.2*</td>
</tr>
</tbody>
</table>

* Data obtained from Gilbert & Taylor (1956)
Other values from Khristov (1970)
Gilbert and Taylor showed in the rat that group A tissues all had the same $^{65}\text{Zn}/\text{zinc}$ ratios at various times when the $^{65}\text{Zn}$ levels were decreasing. The ratio in hepatic, renal and duodenal tissues was 0.05 at 3 days and 0.011 at 34 days. This demonstrates that by 3 days there is complete equilibration between the $^{65}\text{Zn}$ and stable endogenous zinc in these tissues. With regard to $^{65}\text{Zn}$ kinetics this period will be referred to as the 'equilibrium phase' as opposed to 'pre-equilibrium phase' which operates from the time of administration until tissue equilibrium levels are reached.

Table 1-4 Blood and $^{65}\text{Zn}$ concentration of tissue

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Blood volume (μL/g tissue)</th>
<th>$^{65}\text{Zn}$ concentration % of i.v. dose/g after 1 h</th>
<th>3 h</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>178</td>
<td>4.8 3.9 2.9 1.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>481</td>
<td>1.9 1.9 1.7 1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>278</td>
<td>4.0 3.5 2.6 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lewis et al. (1952) Ballou & Thompson (1961)

1-2-5 Cellular and subcellular distribution of zinc

This aspect has been studied using cytochemical staining techniques and autoradiography. Both approaches have shown good agreement. Autoradiography following
intravenous $^{65}\text{ZnCl}_2$ indicated that in the liver, hepatocytes and Kupfferocytes contained $^{65}\text{Zn}$ (Millar et al., 1961). McIsaac (1955) examined $^{65}\text{Zn}$ localisation in the rat pancreatic tissue, and reported a high concentration of $^{65}\text{Zn}$ in the exocrine tissue, in the first 24 h which then rapidly decreased. In contrast, over the four day period of observation $^{65}\text{Zn}$ concentration in the islet tissue increased slowly, and then achieved higher levels than the exocrine tissue. The intravenous dose of zinc, 500 µg per 200 g rat, used in this study is far in excess of the normal levels of zinc absorbed from the diet. These studies using the light microscope were unable to demonstrate any preferential localisation within the cell in either the pancreas or the liver. In the kidney, $^{65}\text{Zn}$ localised more in the cortex than in the medulla (Meschan et al., 1959). In the human prostate $^{65}\text{Zn}$ localises in the holocrine as well as apocrine follicles (Siegel et al., 1961). In the cat prostate, $^{65}\text{Zn}$ localisation has been demonstrated in the apical areas of the acinar cells along the luminal border and in the follicular lumen (Elizabeth, 1970).

The fractional centrifugation method has been used to study the distribution of endogenous zinc as well as $^{65}\text{Zn}$ within the cell. Thiers and Vallee (1957) found that endogenous zinc was present in all the subcellular fractions of rat liver, with highest amounts in the cytoplasm, 43%, and less in nuclei, 37%, microsomes, 12.9%, and mitochondria, 4.4%. $^{65}\text{Zn}$ distributes similarly in the rat liver (Cotzias & Papavasilow, 1964). Subcellular fractions of rat and monkey
prostate gland (Kar & Chowdhry, 1966) showed similar
distribution of \( ^{65}\text{Zn} \), following administration of \( ^{65}\text{ZnCl}_2 \). 
Suso and Edwards (1971) studying the \( ^{65}\text{Zn} \) distribution in
the subcellular fractions of chicken duodenal mucosal
cells found a similarity in the distribution of \( ^{65}\text{Zn} \) after
oral dosing and when \( ^{65}\text{ZnCl}_2 \) is added to the homogenate
of non-dosed birds.

1-2-6 Zinc in blood

Zinc is a constant constituent of blood and human
whole blood normally contains 880 ± 200 \( \mu \text{g} \) zinc/100 ml
(Valee, 1962). In the rat the normal level is 388 ± 22 \( \mu \text{g} 
\) zinc/100 ml (Gilbert & Taylor, 1956). In both species
approx. 12% of the total zinc in blood is found in the
plasma, while most (79%) is in the erythrocytes. Rabbit
erthrocytes, labelled in vivo with \( ^{65}\text{Zn} \), lost the
radioactivity from the intact cell cytoplasm, when they
were suspended in plasma (Dennes et al., 1962). When
equine erythrocytes were incubated in isotonic saline
solutions also containing \( ^{65}\text{ZnCl}_2 \), approx. 30% of \( ^{65}\text{Zn} \)
became localised within the erythrocyte cytoplasm in one
hour (Sivasarma et al., 1959). It was not shown whether
cells suspended in blood plasma containing \( ^{65}\text{ZnCl}_2 \) could
concentrate the isotope to the same extent. Nevertheless,
reversible exchange of zinc may take place between the
erthrocytes and plasma under normal circumstances in
the body.

Gilbert and Taylor (1956), labelled rat serum with
and administered the serum to rats. The $^{65}\text{Zn}$ was cleared from the blood stream. The exponential clearance curve revealed two components ($t_{0.5} = 3.25 \text{ h}$ and $t_{0.5} = 203 \text{ h}$). Molecular associations between zinc and constituents in rabbit plasma have been demonstrated by equilibrium dialysis (Dennes et al., 1962). $^{65}\text{Zn}$ in blood plasma of chickens obtained 2 h after dosage from various routes could not be removed by dialysis against distilled water at $5^\circ$ (Suso & Edwards, 1971). $^{65}\text{Zn}$ binding proteins have been examined in the rabbit serum by $(\text{NH}_4)_2\text{SO}_4$ precipitation and low temperature Cohn fractionation by Dennes et al. (1962), in human serum by starch gel electrophoresis and immunoelectrophoresis (Boyett & Sullivan, 1970), and in chicken plasma by Sephadex chromatography (Suso & Edwards, 1971). Their work has shown that:

(a) Zinc is found in association with a number of plasma proteins.

(b) Zinc is consistently bound to albumin, transferrin and $\alpha_2$-macroglobulin.

(c) The highest amount of serum zinc is bound to albumin, about 60%.

(d) In disease the amount of zinc bound to albumin varies, but the fraction bound to transferrin and $\alpha_2$-macroglobulin remains constant.

The albumin-bound zinc is believed to exchange with tissue zinc. At present there is no conclusive evidence for a specific transport protein for zinc transport like those for Cu and Fe.
Excretion

The removal of $^{65}$Zn from the whole body has been assessed by monitoring the radioactivity remaining in the body at different time intervals after $^{65}$Zn injection. Ballou and Thompson (1961) determined this for the Sprague-Dawley rat after injection of 5μCi of $^{65}$ZnCl$_2$ (= 5 to 10 μg zinc), and found that the radioactivity was removed from the body in an exponential manner. The exponential curve was resolved into two components of biological $t_{0.5}=6.28$ and 215 days.

Irrespective of the route of administration, the gastro-intestinal tract acts as the major route for zinc excretion (Cotzias et al., 1962). The amount of radioactivity recovered in the excreta after intravenous $^{65}$Zn administration has been used as a means of indicating the gross metabolic turnover of zinc (Sheline et al., 1943). The excretion of zinc in the faeces with smaller amounts in the urine has been demonstrated after parenteral administration of $^{65}$ZnCl$_2$ in mice (Cotzias et al., 1962), rats (McIsaac, 1955) and in dogs (Montgomery et al., 1943) and the results are given in Table 1-5. The dose levels in these studies are not comparable, hence, the results cannot be compared accurately. However, a marked species variation in the faecal excretion of $^{65}$Zn is evident. This may also indicate a higher turnover rate of zinc in the lower species. In Khristov's study on rats, the higher faecal excretion (26%) in comparison with McIsaac's figure (16%), may be due to differences in dose levels, variation
<table>
<thead>
<tr>
<th>Animal</th>
<th>Route</th>
<th>Dose (µCi)</th>
<th>Dose (µg zinc)</th>
<th>Faeces (% of dose)</th>
<th>Urine</th>
<th>Collection (Days)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>i.v.</td>
<td>-</td>
<td>0.33-1.6</td>
<td>33</td>
<td>2</td>
<td>2</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>i.v.</td>
<td>150</td>
<td>750</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>(2)</td>
</tr>
<tr>
<td>S.cu</td>
<td></td>
<td>110</td>
<td>-</td>
<td>26</td>
<td>2.6</td>
<td>2</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>i.p.</td>
<td>100</td>
<td>70</td>
<td>63</td>
<td>4.5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>i.v.</td>
<td>-</td>
<td>8 - 13</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>-</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Man*</td>
<td>i.v.</td>
<td>100</td>
<td>1300</td>
<td>3 - 9</td>
<td>2 - 6</td>
<td>7</td>
<td>(5)</td>
</tr>
</tbody>
</table>

(1) Sheline et al. (1943)  
(2) McIsaac (1955)  
(3) Khrystov (1970)  
(4) Wakeley et al. (1960)  
(5) Craig & Siegel (1960)  

*Subjects suffering from malignant disease.
in dietary zinc or to strain differences.

The altered faecal excretion of a test dose of $^{65}$Zn ($^{65}$ZnCl$_2$), after the addition of known amounts of unlabelled zinc to the diet of mice, rats and cattle is shown in Table 1-6. Addition of added unlabelled zinc to the diet increased the faecal excretion of orally administered $^{65}$Zn in rats and cattle. The study in mice using intraperitoneally administered $^{65}$ZnCl$_2$ resulted in an increase in the faecal excretion of $^{65}$Zn. Cotzias et al. (1962) explained this result, by suggesting the presence of a homeostatic mechanism controlling both absorption and excretion of zinc in the gut.

Montgomery et al. (1943), studied the secretion of intravenously injected $^{65}$Zn into the duodenum in dogs. In the first 23 h duodenal aspirates, free of bile and pancreatic juice, obtained from an isolated duodenal loop contained 0.2 to 0.4%, hepatic bile 0.05%, and the pancreatic juice 1.2% of the dose. During the seven day period after the $^{65}$Zn injection the following amounts of $^{65}$Zn were recovered:

(a) Pancreatic juice, 5 to 7%
(b) Bile, 0.4%
(c) Duodenal aspirates, 5%

Birnstingl et al. (1957), in a similar study, confirmed the above findings and also showed that both bile and pancreatic juice contained $^{65}$Zn as early as 15 min following injection.
Table 1-6  Effect of dietary zinc on $^{65}$Zn excretion

<table>
<thead>
<tr>
<th>Animal</th>
<th>Additive</th>
<th>Dietary zinc (p.p.m)</th>
<th>Length of zinc feeding* (days)</th>
<th>Faecal $^{65}$Zn (% of dose)*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse**</td>
<td>ZnSO$_4$</td>
<td>6</td>
<td>5</td>
<td>60</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>5</td>
<td>67 i.p</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>5</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Rat**</td>
<td>Zn(OAc)$_2$</td>
<td>58</td>
<td>28</td>
<td>89</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>175</td>
<td>28</td>
<td>92 oral</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>664</td>
<td>28</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>ZnO</td>
<td>33</td>
<td>17</td>
<td>49</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>7</td>
<td>64 oral</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>600</td>
<td>7</td>
<td>82</td>
<td></td>
</tr>
</tbody>
</table>

*Before $^{65}$Zn administration  * Seven day cumulative faecal excretion

** Figures obtained as (100%) - (% whole body $^{65}$Zn)

(1) Cotzias et al. (1962) (2) Furchner & Richmond (1962) (3) Miller et al. (1970)
The presence of zinc in a variety of metalloproteins with enzyme activity, the requirement of zinc for the catalytic action of another group of enzymes and the importance of zinc in maintaining the structural integrity of certain other macromolecules make this element vital for normal cell function (Vallee, 1952; Prasad, 1966; Parisi & Vallee, 1969 and O'Dell & Campbell, 1970).

Zinc ions are associated with DNA in vivo, and are found in stoichiometric ratios with purified DNA preparations (Mahler & Cordes, 1971). It is believed that Zn$^{2+}$ and other divalent cations such as Co$^{2+}$, Mn$^{2+}$, Cu$^{2+}$, and Fe$^{2+}$ maintain the electrical neutrality of the nucleic acids. Nuclear and extra-nuclear RNA also contain zinc which is responsible for maintaining the correct configuration of the RNA molecule (Wacker & Vallee, 1959).

Glutamate dehydrogenase and malate dehydrogenase, two mitochondrial respiratory chain enzymes, are zinc metalloenzymes. Glutamate dehydrogenase (mol.wt.1,000,000) contains 2 to 6 g-atoms of zinc per mol which is essential for its activity (Adelstein & Vallee, 1958). Malate dehydrogenase (mol.wt.40,000), of bovine cardiac muscle contains 1 g-atom of zinc per mol (Harrison, 1963). Dunn et al. (1969) found that horse alcohol dehydrogenase (mol.wt. 80,000) contains 3.1 to 4.3 g-atoms of zinc per mol and that removal of the zinc causes denaturation. Alkaline phosphatase also contains zinc, and is present...
in high levels in bone, intestinal mucosa, kidney
(Stadtman, 1961) and most body fluids. A zinc requiring
alkaline phosphatase has been found in the ventral lobe
of the rat prostate (Hopkin & Wakely, 1961). Recently, a
zinc containing alkaline phosphatase has been isolated
from leucocytes (Dechatelet et al., 1971). Carbonic
anhydrase (mol.wt. 30,000), containing 1 g-atom of zinc
per mol has been isolated from mammalian erythrocytes
(Keilin & Mann, 1940 and Duff & Coleman, 1966). Pancreatic
carboxypeptidase-A and B, (mol.wt. 34,000) containing 1 g-atom
of zinc per mol has been isolated from the bovine and porcine
pancreas (Vallee & Neurath, 1954 and Wintersberger et al.,
1962).

\( \text{Zn}^{2+} \) has been shown to activate several other enzymes
in vitro such as glycylglycine dipeptidase, arginase,
histidine deaminase, and oxaloacetate decarboxylase

In several of these enzymes zinc is known to function
as a part of the active site by linking the substrate and co-
enzyme to the protein. It achieves this by causing alterations
in the tertiary structure of the protein by bringing the
groups that constitute the active centre into close proximity.
This has been demonstrated for carboxypeptidase-A (Vallee,
1964) and carbonic anhydrase (Coleman, 1967). The role
of Zn in carboxypeptidase as postulated by Vallee (1964)
is given in Fig. 1.

Zinc also stabilizes the quaternary structure of
horse liver alcohol dehydrogenase (Oppenheimer et al., 1967).
Zinc is therefore important in the enzymes concerned with digestion, cellular respiration, gaseous transport and intracellular energy metabolism.

Zinc containing metallo-proteins such as metallothionein (Pulido et al., 1966) and cytocupreins (Carrico and Deutsch, 1970) with as yet undefined biochemical functions have been isolated and purified.

Fig. 1. The active centre of carboxypeptidase.

$\text{Zn}^{2+}$ is bound to cysteine sulphur atom ($S^-$) and asparagine nitrogen atom ($N$) of the enzyme. $A$ and $B$ are proton and electron donor groups of the enzyme. The binding of a terminal dipeptide to $\text{Zn}^{2+}$ and to the protein moiety of the enzyme is shown.
Zinc is required for the crystallisation of insulin but there is no evidence available to suggest a zinc requirement for insulin action. Blundell, Hodgkin et al. (1971) noted that insulins from animals other than guinea-pig and coypu are stored as zinc-insulin hexamers in the granules of the β-cells of the pancreatic islets. Therefore zinc may be required for the polymerisation of the insulin molecule for storage.

The high content of zinc in the α-cells of duck pancreas, and the production of more glucagon in this species than in other vertebrates led Weitzel et al. (1956) to suggest a zinc-glucagon relationship similar to that of zinc and insulin.

Flynn et al. (1972) studying the effect of zinc on in vitro corticosterone production by rat adrenal slices found that chelation of zinc with dimethyl thiocarbamate lowered the production of this hormone. They also showed that zinc ions form complexes with ACTH. The requirement of zinc in the function of gonadotrophin and growth hormone has been proposed.

1-2-9 Zinc and the malignant cell

Zinc is essential for the normal proliferative process (Westmoreland, 1971) and is also required for tumour growth (Petering et al., 1967). Following injection, $^{65}Zn$ accumulates preferentially in spontaneous $\beta$H mammary-adenocarcinoma and dibenzanthracene-induced spindle cell sarcoma of mice (Heath & Liquier-Milward, 1950). The presence of melanoma in mice reduced the loss of intraperitoneally
injected $^{65}$Zn from the body. The control animals excreted more than 60% of the dose and the tumour bearers less than 50%, in 13 days (Prasad et al., 1969). Experimental zinc deficiency in rats caused a marked growth retardation of the transplanted Walker carcinosarcoma and increased the survival time (McQuitty et al., 1970).

In humans low serum zinc levels have been reported in bronchial carcinoma (Davies et al., 1968), leukemia and Hodgkin's disease (Dunn et al., 1969). In benign prostatic hyperplasia zinc concentration in the affected areas of the prostate gland has been found to be increased above the amount of zinc in histologically recognisable normal adjacent tissue (Schrodt et al., 1964). Siegel et al. (1961) showed an increased uptake of $^{65}$Zn in the benign hyperplastic prostate gland in comparison with normal gland.

Increased rate of mitosis, and a demand for increased protein synthesis, may be the cause of increased zinc requirement by the tumour cells.

1-2-10 The use of radioactive zinc in scanning for tumours of the pancreas and prostate gland

Zinc in its radioactive forms has been utilized for pancreatic and prostatic scanning by gamma-ray scintigraphy. Of the radionuclides of zinc, $^{62}$Zn ($t_{0.5} = 9.2$ h) and $^{69m}$Zn ($t_{0.5} = 13.8$ h) are preferred for clinical use owing to their short physical half-lives and suitable gamma-ray emissions.
$^{65}$Zn with a long physical half-life (265 days) and a high penetrating gamma-radiation (1.1 MeV), presents a health risk. It was used, however, for clinical trials until the new nuclides became available.

The high concentration of $^{65}$Zn found in the pancreas of several experimental animals after intravenous injection of $^{65}$ZnCl$_2$ has been the basis for selecting $^{65}$Zn for pancreatic scanning. Meschan et al. (1959) using 100 μCi of intravenous $^{65}$ZnCl$_2$ failed to visualise the pancreas of the dog, although external counting over the pancreatic area showed 20% of the dose administered. However, biopsy samples of pancreas contained only 3-6% of the dose, during 2 to 8 h. This implies that approx. 14-17% of the radioactivity detected over the pancreatic area originated in other tissues, principally in the liver. Cottrall and Taylor (1970) utilised $^{62}$Zn, a less penetrating, lower energy (0.511 MeV) gamma-ray emitter and concluded that the levels of radioactivity present in the abdomen of humans 1-8 h after 250 μCi of intravenous $^{62}$ZnCl$_2$ was so great as to preclude the use of $^{62}$Zn for pancreatic scanning. During this period radioactivity in the liver was estimated to be approx. 44% of the dose. The stimulation of pancreatic secretion in the rat, by pancreozymin or secretin or the complexing of zinc by glycine or tryptophan failed to alter the pancreas/liver ratio of $^{62}$Zn to an extent sufficient for clinical work (Cottrall & Taylor, 1970).

The high levels of zinc detected in the prostate gland in normal and hyperplastic conditions have spurred
the use of radionuclides of zinc for visualisation of this gland. In benign prostatic hyperplasia zinc content and the $^{65}$Zn uptake by the prostate gland is increased, but in carcinomatous changes of the prostate, a distinct fall in the zinc content and reduced uptake of $^{65}$Zn has been reported (Rossoff & Spencer, 1965 and Gyorkey et al., 1967). On the basis of these observations $^{69m}$ZnCl$_2$ (Johnston et al., 1968) has been used to distinguish carcinoma and other prostatic diseases. High concentrations of $^{65}$Zn in the prostate gland of humans following intravenous $^{65}$Zn has been shown to occur only after about two weeks (Spencer et al., 1965 and 1966), but such time intervals are not suitable for scanning with $^{62}$Zn or with $^{69m}$Zn due to rapid radioactive decay of these nuclides. Also, a long delay in a diagnostic procedure is not acceptable and therefore, methods of augmenting prostatic uptake of nuclides of zinc have been attempted. Johnston et al. (1968) administered pilocarpine to dogs to induce prostatic fluid flow and Prout et al. (1959) treated dogs with testosterone propionate and demonstrated enhanced $^{65}$Zn uptake by the prostate gland of the dog. These agents have not been reported to be used in patients.

Because of the lack of specific tissue localisation, nuclides of zinc have not been successful as scanning agents in clinical diagnosis of pancreatic and prostatic disease.

1-2-11 The use of oral zinc sulphate supplementation in medicine

The observation that porcine parakeratosis was induced
by feeding a low zinc diet (Vallee, 1962) and that a skin injury in a zinc-deficient calf failed to heal for a long period (Miller et al., 1965) led to further work suggesting a role for zinc in wound healing. Savlov et al. (1962) demonstrated that $^{65}$Zn accumulated at the site of skin and muscle repair and a similar observation for the healing of experimental arterial wounds was reported by Strain et al. (1968). These observations led to the administration of ZnSO$_4$ to assist in wound healing as reported by Greaves and Skillen (1970) and Lavy (1972). Greaves & Skillen (1970) administered 660 mg. ZnSO$_4$$\cdot$7H$_2$O orally, per day over a period of months to patients with venous leg ulcers and demonstrated satisfactory healing. The dose was approx. 60 times the normal daily zinc intake and plasma zinc levels were 25% above the normal in the majority of patients after 16 weeks of therapy, but no abnormalities were observed in liver function tests. In the study on rats Lavy (1972) administered 20 mg. ZnSO$_4$$\cdot$7H$_2$O orally for 13 days after abdominal surgery and reported improved wound healing.

Fell et al. (1973) administered $^{65}$Zn intravenously 20 days prior to total hip replacement in humans and demonstrated increased urinary $^{65}$Zn and stable zinc excretion post-operatively which they claimed was derived from muscle tissue. In 1971, Westmoreland showed that connective tissue regeneration required zinc. The increased excretion of zinc after trauma or the increased zinc required for regenerative processes are possibly compensated for by the
ZnSO$_4$ therapy. Oral zinc has also been reported to be beneficial in cases of zinc malabsorption, growth failure, retarded development, 'hypogonadal dwarfism' (Prasad, 1966) and in porphyria.
1-3 Gallium

1-3-1 Introduction

Gallium first came to the attention of Mendeleéff in 1871, who correctly predicted its occurrence and also outlined some of its physical and chemical properties even before it was discovered. Gallium was first isolated by Boisbaudran from a zinc ore in 1875. It ranks 57th among the elements in abundance on the earth's crust and occurs at a concentration of approx. \(10^{-11}\) per cent. Its presence in plants and animals and man has been noted by several workers including Belozerov (1965). Whether trace amounts of gallium have a biochemical role or whether it is an anutrient has not been reported.

1-3-2 Absorption, tissue distribution and excretion

Enteric absorption of gallium is poor. Dudley and Levine (1949) showed this by feeding rats with gallium chloride, gallium lactate and gallium citrate in the diet at a level of 1000 p.p.m gallium. Only traces of gallium have been detected in the bone and almost none in tissues such as the liver kidney and spleen. In the laboratory rat, tissue distribution studies following intravenous injection of stable gallium, \(^{72}\text{Ga}\) containing stable gallium and carrier-free \(^{67}\text{Ga}\) have been reported.

Most inorganic salts of gallium are known to hydrolyse at physiological pH, in aqueous solution. Hence, \(\text{GaCl}_3\) dissolved in 15% to 20% citric acid solution has been used for tissue distribution studies and clinical trials (Brunner et al., 1953, and Perkinson et al., 1953). It is believed that a complex is formed by mixing \(\text{GaCl}_3\) with citric acid, which will be referred to as gallium citrate and this has the following
properties:

a) The citrate moiety maintains Ga\(^{3+}\) in solution,
b) The citrate is metabolisable,
c) The citrate is believed to sustain the release of Ga\(^{3+}\) following administration,
d) The gallium citrate is stable under conditions of sterilisation by autoclaving (120°C at 15 p.s.i.)

Brunner et al. (1953) using \(^{67}\text{GaCl}_3\) in 15% citric acid solution studied the tissue distribution of \(^{67}\text{Ga}\) in the rat. The \(^{67}\text{Ga}\) preparation did not contain stable carrier gallium above the limit of detection (2 x 10\(^{-7}\) g gallium/ml).

Following the intravenous administration of trace quantities nearly 45% of \(^{67}\text{Ga}\) was retained in the body for 20 days. At 24 h, 79% of \(^{67}\text{Ga}\) was retained in the body of which approx. 21% was localised in the liver and 26% in the skeleton.

Over the 20 days no appreciable quantities were lost from the skeleton but the liver lost most \(^{67}\text{Ga}\). When stable gallium was added to the trace dose of \(^{67}\text{Ga}\) the amount of \(^{67}\text{Ga}\) retained in the body was decreased. For example, 5 days after an intravenous dose of \(^{67}\text{Ga}\) containing 2.5 x 10\(^{-3}\) g gallium/kg, 60% of the \(^{67}\text{Ga}\) was retained whereas only 18.5% was retained when the carrier level was raised to 25 mg gallium/kg. The decreased retention of \(^{67}\text{Ga}\) was observed in both liver and skeleton although the effect in the latter was less marked. Similar results were obtained with \(^{72}\text{Ga}\) indicating that there was no discrimination between isotopic forms of gallium and that the results were due to the different dose levels used.
The results of these studies are summarised in the Table below:

Table 1-7  The effect of carrier stable gallium on $^{72}\text{Ga}$ citrate and $^{67}\text{Ga}$ citrate distribution in the rat

<table>
<thead>
<tr>
<th>Gallium concentration (mg/kg)</th>
<th>26</th>
<th>C.F.*</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclide</td>
<td>$^{72}\text{Ga}$</td>
<td>$^{67}\text{Ga}$</td>
<td>$^{67}\text{Ga}$</td>
</tr>
<tr>
<td>Day of sacrifice after injection</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Number of animals</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Tissue radioactivity**:-

<table>
<thead>
<tr>
<th>Tissue</th>
<th>26 C.F.*</th>
<th>25 C.F.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.91</td>
<td>5.81</td>
</tr>
<tr>
<td>Skeleton</td>
<td>20.9</td>
<td>21.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.07</td>
<td>1.66</td>
</tr>
<tr>
<td>Blood</td>
<td>0.14</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Reference

1  2  2

* Carrier-free

** as a per cent. of injected dose in the whole organ

References:-  
1. Brunner et al., 1953 a
2. Brunner et al., 1953 b
Four hours after the intravenous administration of gallium high concentrations were observed in the kidneys but the gallium levels decreased rapidly due to urinary excretion. With tracer doses of carrier-free $^{67}\text{Ga}$ similar amounts of radioactivity were excreted in both urine and faeces, but the presence of carrier gallium above 0.25 mg/kg, increased the urine/faeces ratio, reaching a value of 10 at a carrier level of 25 mg gallium/kg.

1-3-3 $^{67}\text{Ga}$ plasma protein binding

$^{67}\text{Ga}$ binding by constituents of blood has been studied by Hartman and Hayes (1969). In an in vitro system with rabbit serum labelled with $^{67}\text{Ga}$ citrate containing known amounts of stable gallium varying between $1.0 \times 10^{-3}$ pmol to 10.0 pmol, they established the following facts:

a) The percentage $^{67}\text{Ga}$ citrate bound by rabbit serum decreased at concentrations above $3.6 \times 10^{-2}$ pmol gallium/10 ml of serum. Below this concentration $^{67}\text{Ga}$ was bound to an extent of over 91%.

b) The absolute amount of $^{67}\text{Ga}$ bound increased up to 1.4 pmol gallium/10 ml, after which $^{67}\text{Ga}$ binding was lowered.

c) $^{67}\text{Ga}$ binding was not affected by citrate up to a concentration of 6.6 pmol citrate/10 ml serum.

d) The percentage of $^{67}\text{Ga}$ bound, was not affected.
between pH 7.5 and 8.0 but below pH 7.4 binding decreased.

e) The gallium binding components in plasma at a concentration of $1.0 \times 10^{-3}$ µmol gallium/10ml serum are not ultrafiltrable through a membrane with a mol.wt. cut-off of 10,000.

f) $^{67}$Ga was eluted in association with 280nm absorbing molecules excluded from Sephadex G-25 gel.

g) The percentage binding obtained by gel-filtration (approx. 60%) was lower than that obtained by equilibrium dialysis (90%).

Ito et al. (1970) using Sephadex G-75 gels chromatographed blood sera obtained from rabbits following intravenous $^{67}$Ga citrate and found also that $^{67}$Ga was eluted in association with proteins. Although these studies indicate that gallium interacts with serum proteins, the binding proteins were not identified.

1-3-4 Subcellular Localisation of $^{67}$Ga

Ito et al. (1970) and Becker et al. (1972) studied the distribution of $^{67}$Ga in the cell organelles of rabbit and rat liver following intravenous injection of $^{67}$Ga citrate. The soluble as well as particulate fractions contained gallium with the highest amount found in the soluble and nuclear fractions. Light microscopic autoradiography showed a high $^{67}$Ga localisation in macrophages of
the lymphoreticular tissue and also Kupffer cells and polygonal cells in the liver of rats (Swartzendruber et al., 1970). Electron microscopic autoradiography showed $^{67}$Ga localisation in lysosomes, in lysosome-rich cells such as the macrophages, hepatocytes and the proximal convoluted tubular cells of the rat kidney (Swartzendruber et al., 1972).

1-3-5 The affinity of cancer cell for gallium

$^{67}$Ga localisation in a number of transplantable tumours in mice and rats (Hayes et al., 1970) and rabbits (Ito et al., 1971) have been reported. Hayes et al. (1970) reported $^{67}$Ga localisation in spontaneous mammary tumours of C3H mice and in hyperplastic thymus of the AKR/J mice which became spontaneously leukemic. Ito et al. (1971) showed that more $^{67}$Ga was located in the nuclear fraction than in the soluble fraction of tumour cells from VX-2 epidermoid carcinoma of rabbit. Similar results were obtained by Bickel and Hanson (1972) with JBL plasmocytoma ascites cells of rats and they also demonstrated that these cells concentrated gallium during the rapid growth phase. This is in agreement with the findings of Hayes et al. (1970) and Nash et al. (1972) that $^{67}$Ga concentrates mainly in viable tumour cells rather than in necrotic zones. The suppression of tumour growth by radiotherapy (Vaidya et al., 1970) and chemotherapy (Heidenreich et al., 1972) decreased the uptake of $^{67}$Ga by the tumour. The affinity of the tumour cell for gallium was also shown by Hart et al. (1972)
who observed an antitumour effect of Ga(NO₃)₃·9H₂O in a number of solid tumours grown in mice and rats.

1-3-6 The use of ⁶⁷Ga as a tumour scanning agent

Compounds of gallium have rarely been considered in diagnostic or therapeutic medicine but Dudley and Maddox (1949) envisaged its application in the form of ⁷²Ga citrate as a radio-therapeutic agent in the suppression of osseous tumours in the bone. In clinical trials Edwards & Hayes (1969 & 1970) used cyclotron-produced ⁶⁷Ga citrate and showed an affinity of gallium for soft tissue tumours. In later trials a variety of tumours including bronchial carcinoma, gastric carcinoma and malignant melanoma have been shown to concentrate ⁶⁷Ga (Ito et al., 1971, Lavender et al., 1971 and Langhammer et al., 1972). In Hodgkin's disease ⁶⁷Ga accumulated in the lymph nodes (Edwards & Hayes, 1970). ⁶⁷Ga Localisation in inflammatory lesions was initially reported by Lavender et al. (1971). Recently, Blair et al. (1973) observed gallium localisation in experimental staphylococcal abscesses in the rat and suggested that ⁶⁷Ga citrate may be of use in locating deep seated abscesses in man. ⁶⁷Ga citrate is not therefore specific for tumours only. The mechanism of ⁶⁷Ga localisation in tumours or abscesses is not known.

1-3-7 Toxicity of gallium

Dudley et al. (1950) gave the following results.
for the ten day LD$_{50}$, following subcutaneous gallium citrate.

<table>
<thead>
<tr>
<th>Species</th>
<th>LD$_{50}$ (mg Ga/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>600</td>
</tr>
<tr>
<td>Rat</td>
<td>100</td>
</tr>
<tr>
<td>Rabbit</td>
<td>45</td>
</tr>
<tr>
<td>Dog</td>
<td>15</td>
</tr>
</tbody>
</table>

These results show that gallium toxicity is species dependent with a higher toxicity in the higher animals. Gallium chloride and gallium lactate in the diet of rats at a level of 15 to 20 mg gallium per day up to 26 weeks failed to manifest any toxic symptoms and Dudley and Levine (1949) concluded that gallium compounds such as the chloride, lactate and citrate offer little hazard as an ingested poison. Intravenous gallium lactate, at a dose level of 20 mg Ga/kg body weight produced toxic symptoms in the rat. In this species toxicity of gallium citrate is less than gallium lactate as shown by the ten day LD$_{50}$: 220mg Ga/kg body weight for gallium citrate and 46 mg Ga/kg body weight for gallium lactate (Brunner et al., 1953). Acute intravenous dosing of gallium citrate in rats (220 mg Ga/kg), in dogs (35 mg Ga/kg) and chronic dosing (2.5 mg Ga/kg) twice a week in the rat for a long
period produced lesions in the kidney, lymph nodes and spleen in both species. Polymorphonuclear lymphocyte infiltration in the lymph nodes was pronounced (Brunner et al., 1953). Areas of high lymphocyte infiltration contained little gallium. Symptoms like those due to common metal poisoning such as diarrhoea, albuminuria, anorexia have been noticed in experimental gallium poisoning in the above studies. Although a knowledge of gallium poisoning is of interest, toxicity due to carrier-free doses of $^{67}$Ga, $4 \times 10^{-10}$ μmol $^{67}$Ga/human, used in tumour scanning, is unlikely to occur.
1-4 Aims of the present work

From the review given in this Chapter, it is clear that the basic outlines of the metabolism of zinc have been worked out. The accumulated evidence in this field is now sufficient to allow one to seek more precise information on the metabolism of this trace element at the cellular level, with a clearer insight into the absorption, transformation (i.e. incorporation into other molecules) and removal from the cell. It is believed that such investigations should throw light on its role in life-processes in health and disease. A thorough understanding of the behaviour of zinc may be of importance in the development of radio-zinc-pharmaceuticals, whose differential absorption would enable scanning of organs and tissues with increasing accuracy and precision.

Initial studies utilised the then available $^{65}\text{Zn}$ preparations which in most instances contained a high level of stable carrier zinc. It appears that the doses of zinc used in experiments with small animals were in excess of physiological amounts, and hence the stable carrier zinc would have influenced the observed behaviour of $^{65}\text{Zn}$. Therefore, a high specific activity $^{65}\text{Zn}$ preparation was utilised to obtain sub-physiological zinc doses, which were used to investigate further the tissue distribution, metabolism and excretion of zinc.

$^{67}\text{Ga}$ is a new radio-diagnostic agent which is used in clinical trials for tumour scanning. Knowledge of the behaviour of gallium in the body is scant and therefore the tissue distribution and excretion of gallium have been investigated.
CHAPTER 2

EXCRETION OF ZINC

2-1 Introduction

The pharmacokinetic aspects of $^{65}\text{Zn}$ have been reviewed in Chapter 1. Most of these studies by other workers have utilised high levels of non-radioactive zinc accompanying the trace dose of $^{65}\text{Zn}$. It is conceivable that the carrier zinc influenced the observed behaviour of $^{65}\text{Zn}$ which therefore may not have behaved physiologically. $^{65}\text{Zn}$ doses in this study contained minute amounts of carrier zinc and can be considered as sub-physiological amounts.

This study was conducted to reveal the excretion of sub-physiological doses of $^{65}\text{Zn}$ and consisted of five fundamental investigations:

Whole body counting,
Faecal excretion,
Urinary excretion,
Faecal and urinary excretion of $^{65}\text{Zn}$ when Additional oral ZnSO$_4$ is given
Biliary and pancreatic excretion.

2-2-1. Animal maintenance

Male Wistar strain rats were used and were obtained from the Royal Postgraduate Medical School animal house, four weeks prior to their use. Normal healthy rats were selected of weight 230 ± 25 g at 11 - 12 weeks of age.

During the four weeks in the laboratory the rats were kept in a small room with adequate ventilation at a temperature of 70 - 72°F. Although the humidity was not controlled, occasional checks showed that the relative humidity was about 50%. Rats were exposed to 12 h light periods (9 a.m. - 9 p.m.).

During the pre-experimental period the rats were housed in plastic cages (NKP Cages, Kent). The cages had stainless steel roofs and wire floors which were elevated to prevent body contamination of radioactive excreta after dosing.

Groups of four animals were trained for a period of four weeks to semi-restrained conditions in metabolism cages, which were made of perspex and contained a cylindrical living compartment of dimensions 20 x 30 cm, with a perspex lid. The floor was made of stainless steel wire coated with perspex. A removable perspex separator was fitted to allow separation of solid and liquid excreta.

During the $^{65}$Zn studies the cages were cleaned and decontaminated by washing the whole cage in tap water. The separators were treated with 1% EDTA solution and subsequently dipped in 1% ZnSO$_4$ solution. This procedure was adopted to minimise $^{65}$Zn contamination.

-40-
Other animals were trained to acute restraint, four weeks prior to bile duct cannulation. The standard restraining cages were constructed out of perspex with perspex coated wire floors and roofs.

Animals in all restraining cages were removed from the cage once a day, during the pre-experimental as well as the experimental period, and allowed to clean themselves.

Rats were given a pellet diet 41B (Dixon & Son Ltd., Herts.). The food hoppers were filled at 7 p.m. and the remaining food was removed at 9 a.m. next day. The diet was cited by the manufacturer to contain the following mineral composition:

<table>
<thead>
<tr>
<th>Element</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( µg/kg diet )</td>
</tr>
<tr>
<td>Ca</td>
<td>$6.1 \times 10^6$</td>
</tr>
<tr>
<td>Mg</td>
<td>$1.7 \times 10^6$</td>
</tr>
<tr>
<td>Fe</td>
<td>$6.1 \times 10^4$</td>
</tr>
<tr>
<td>Cu</td>
<td>$6 \times 10^3$</td>
</tr>
<tr>
<td>Zn</td>
<td>$15 \times 10^3$</td>
</tr>
<tr>
<td>Mn</td>
<td>$35 \times 10^3$</td>
</tr>
<tr>
<td>Co</td>
<td>73</td>
</tr>
</tbody>
</table>

The daily consumption of solid food in a group of six rats weighing 230 ± 5 g was checked. These consumed 24 – 30 g of food per night which represents a daily intake of zinc of 350 – 450 µg.
2-2-2 Anaesthesia

Anaesthesia was performed using diethyl ether, containing $3 \times 10^{-4}$% pyrogallol as an antioxidant (Hospital Pharmaceutical Dept.). Rats were made to inhale ether vapour by keeping them in a stream of air bubbled through ether. Isotope injection was carried out under light ether anaesthesia, indicated by a respiratory rate of 100 - 110 per min. In bile duct cannulation, an operation which takes approx. 45 min, rats were kept under fairly deep anaesthesia, and a respiratory rate of 90 - 100 per min was maintained by 1 - 2 min exposure to ether at approx. 10 min intervals. In tissue distribution studies animals were deeply anaesthetised, with a respiratory rate of less than 70 per min prior to sacrifice. Wherever possible ether inhalation was kept to a minimum.

2-2-3 Cannulation of the bile duct

In Wistar rats visual examination shows that the bile duct is embedded in the distal four fifths of the pancreatic tissue. In this study, the anatomy of the pancreatic ducts with regard to the opening into the common bile duct was ascertained as follows:

(a) The bile duct was ligated about half a centimetre below the hilus of the liver, at a point where the pancreatic tissue no longer surrounds the bile duct.

(b) Indian-ink was gently injected from the duodenal end of the common bile duct.

(c) Pancreatic tissue was carefully excised from the omentum and freed from the overlying adipose tissue.
by immersing the preparation in a mixture of acetone: ether (1:1).

(d) The fat-free preparation was stained with 1% eosin solution and flattened out on a glass plate.

The ink entered the pancreatic tissue through at least two large ducts and a number of small ducts (see Fig. 2.1). Fig. 2.2. shows the positions where cannulae were inserted in the bile duct for the collection of either bile or bile and pancreatic fluid.

Cannulations were performed between 8 - 10 a.m. on animals starved for 24 h before the operation. A polythene cannula of i.d. 0.028 cm and o.d. 0.061 cm (Portex, Kent), cleaned free of foreign particles with 95% ethanol, was bevelled and smoothed by heat at one end. An area on the right ventro-lateral aspect, below the rib-cage of the animal was closely clipped and cleaned with cotton wool soaked with methanol. An oblique incision 2.5 - 3.0 cm, long was made from the xiphisternum towards the lateral line. The duodenal loop was moved posteriorly to visualise the bile duct. The bile duct was held anterior to the pancreatic tissue with thin silk thread (Mersilk) and the duct gently pulled back with the free ends of the thread. While holding it in position, an incision was made on the bile duct and the bevelled end of the cannula was guided into it with the aid of fine forceps. The cannula was secured in position with two knots on either side of the incision. For the collection of combined bile and
Fig. 2.1. The passage of Indian-ink through the bile duct into the pancreas of a Wistar rat. The dark areas in the pancreas are due to the localisation of ink. The arrows indicate the duodenal and hepatic ends of the bile duct.
Fig. 2.2. Diagram to show the positions where cannulae were inserted to obtain bile (position A) and bile and pancreatic fluid (position B) in the common bile duct of the Wistar rat.
pancreatic fluid, the bile duct was ligated at a point very near the duodenum. The cannula was guided about half a centimetre along the bile duct and secured.

For both procedures, the free end of the cannula was guided to the exterior through the lumen of a gauge 19 injection needle introduced into the peritoneal cavity from a mid-dorsal position of the animal. The pancreatic tissue was irrigated with sterile, 0.9% NaCl solution at 37°. The surplus solution was later removed by tilting the animal to its right side. This procedure was adopted to wash bile and pancreatic fluid from the areas contaminated with these fluids, which happened when the bile duct was incised. The opening of the abdomen was sutured in three layers and the area was sealed with a surgical spray, Nobecutane (B.D.H.).

A supplement of 2.5% glucose and 0.9% NaCl was added to the drinking water of the cannulated animals. Pellet food was given in the normal manner.

During the operative procedure care was taken to:

(a) Avoid handling the liver, the pancreas and duodenum.

(b) Avoid application of undue pressure on these organs due to excess leverage on the bile duct.

This procedure prevented exposure of abdominal viscera. The post-surgical recovery appeared to be very satisfactory. At the termination of the study, four days later, histological sections of the liver showed bile duct
proliferation, but no signs of ascending infection or inflammatory reactions of the liver were recorded.

The rats were allowed to recover for 24 h before experimentation.

2-2-4 Method of injection

The test compounds were injected into the right lateral caudal vein (i.v.) using a gauge 20 needle. The radioactive solution was gently forced into the vein over a period of 2 min. A volume of blood equal to the total injectate was withdrawn and slowly fed back into the vein. The injection area was washed to remove any radioactivity. If extravasation occurred, as indicated by poor blood flow into and out of the syringe, the animals were not used for further experimentation. Failures due to imperfect injections were about 10%.

2-2-5 Sample collection

Faeces and urine were obtained from the animals kept in metabolism cages, the daily faecal samples being collected in polythene bags. Faeces were then dried in an oven at 120°C for 24 h on a watch glass. These were transferred in one gram amounts into counting vials.

Urine samples were collected directly into weighed counting vials and at the end of each 24 h period the vials were reweighed, and one gram amounts were placed into separate vials for radioactivity measurements.

Bile, and bile and pancreatic fluid samples were collected hourly in weighed polythene containers
in an automatic fraction collector. Precautions were taken to minimise evaporational losses from the containers by using caps perforated in two places to introduce the bile duct cannula and to allow the passage of air.

2.3  Radioactivity measurement

2.3.1 Ionisation chamber

A high pressure ionisation chamber (Twentieth Century Electronics Ltd., Surrey) was used to measure the radioactivity of stock solutions, in order to ascertain the specific dilutions required for the calibration of the more sensitive whole body counter and the highly sensitive gamma-ray spectrometer as well as for determining the dilutions for biological work. The ionisation current produced by 1 - 2 mCi of $^{65}$Zn and $^{67}$Ga contained in gravimetrically known quantities of solution were measured and the radioactivity in $\mu$Ci was obtained from the following equation:

\[
\frac{(i_x - b)}{(i_{Ra} - b)} \times \frac{K_{Ra}}{K_x} \times 1000 = \mu\text{Ci}
\]

Where,
- $i_x$ = ionisation current in pA due to nuclide $x$,
- $i_{Ra}$ = ionisation current in pA due to 1.0 to mCi of $^{226}$Ra,
- $b$ = background current in pA,
- $K_x$ = specific ionisation constant of nuclide $x$ in R/h/mCi

\[
K_{Ra}, (^{226}\text{Ra}) = 8.4 \\
K_{Zn}, (^{65}\text{Zn}) = 2.7 \\
K_{Ga}, (^{67}\text{Ga}) = 1.55
\]
2-3-2 Animal whole body counter

This instrument consists of eight Geiger-Müller tubes, a probe unit and a scaler. The Geiger tubes are arranged in a circle surrounding a central perspex platform, on which is placed the radioactive source.

The animals following radioactive dosing, were restrained in a 400 cm³ polythene box and placed in the centre of the platform. The probe unit was set to impose a paralysis time of 200 µs. The observed counting rates were corrected for the counts lost during the imposed paralysis time, using a chart constructed according to the formula:

\[
N = \frac{N_0}{1 - N_0 t}
\]

where,

- \( N_0 \) = observed count rate,
- \( N \) = corrected count rate,
- \( t \) = paralysis time.

The true counting rate of the source was obtained by the subtraction of the background counting rate. The instrument was calibrated using standard quantities of the appropriate radionuclide, and was used to record counting rates between 100 - 500 c.p.s. The stability and the performance of the counter was checked daily using a sealed source of 20 µCi of \(^{65}\text{Zn}\).

2-3-3 Gamma-ray spectrometer

The radioactivity of tissues and other biological extracts were assayed in a gamma-ray spectrometer (Nuclear Enterprises, Edinburgh). The gamma counting system
consists of a cylindrical NaI crystal viewed by two photomultiplier tubes in summation.

Prior to use in each series of measurements the instrument was calibrated as follows:

(a) The full gamma-ray spectrum was obtained for the nuclide.

(b) The counting rate of a sample of known radioactivity was obtained using a suitable area of the spectrum.

The background radioactivity was checked using empty vials under similar counting conditions. Although the instrument was later modified to provide adequate shielding around the detector, when $^{65}$Zn was counted for the experiments described here the background count rate increased owing to other samples in the magazine. This was minimised by keeping a blank between each vial containing a radioactive sample. For $^{67}$Ga, a lower energy gamma-ray emitter, blank vials were kept only at the beginning and at the end of the magazine. Routinely, samples of approx. 1 g were counted, but on occasions where larger samples were used the counting rates were corrected for the change in geometry.

Counting times of 100 s were adequate for tissue counting, at the activity levels used, at all times. Biological extracts were counted for periods of 100 - 1000 s and the background was counted for a similar length of time. Paralysis time corrections were not applied in all these estimations as the counting rates were low.

The instrument was calibrated using standard quantities
of the appropriate radionuclide. Wherever applicable radioactive decay was taken into consideration and the appropriate corrections were made using decay charts.
Materials

$^{65}$Zn

$^{65}$Zn was produced in the M.R.C. cyclotron according to the nuclear reaction:

$$^{65}_{29}Cu + ^{2}_{1}D_1 ^ {65}_{30}Zn + ^{2}_{0}ln$$

(Clark et al., 1967)

The activated Cu target was processed by the Radiochemical Centre (Amersham), to recover $^{65}$Zn. $^{65}$Zn, with a specific activity better than 100 mCi/mg zinc, was supplied in glass bottles, each containing 5 mCi of $^{65}$ZnCl$_2$ in 10 ml of 0.1M-HCl.

A dose of 20 µCi was used in all experiments. The stock solutions were diluted by the addition of the required volume of 0.1M-HCl to contain 20 µCi $^{65}$Zn in 0.2 ml.
2-5 Experimental results

2-5-1 Whole body counting

Three rats were injected i.v. with a dose of 20 μCi of $^{65}$ZnCl$_2$ ($= 0.2$ μg zinc). Upon recovery from anaesthesia, the rats were transferred to polythene boxes for radioactivity counting and were later housed in metabolism cages. Radioactivity in the animals was counted individually, in the whole body counter, each morning for a period of 5 weeks. The contamination of polythene boxes was prevented by polythene sheets which were replaced daily.

<table>
<thead>
<tr>
<th>Day</th>
<th>% $^{65}$Zn retention in rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0*</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>84.0</td>
</tr>
<tr>
<td>2</td>
<td>79.5</td>
</tr>
<tr>
<td>3</td>
<td>71.0</td>
</tr>
<tr>
<td>4</td>
<td>68.2</td>
</tr>
<tr>
<td>5</td>
<td>66.9</td>
</tr>
<tr>
<td>6</td>
<td>62.5</td>
</tr>
</tbody>
</table>

*the initial count rate was assumed to be 100%.

The radioactive counting was done for a period of 100 s and was repeated five times for each animal.
The counting rate observed for each animal immediately after the injection was considered as 100%, and the subsequent estimates were expressed as a percentage of this value.

This experiment was repeated using a second group of three rats. The amounts of $^{65}$Zn retained in the body of individual rats during the first 6 days are shown in Table 2-1. The six animals in this study retained somewhat similar amounts of $^{65}$Zn, i.e., individual variations were small.

These estimations were combined for a total of 36 days and the mean values obtained from the six animals are represented graphically in Fig. 2.3. The whole body retention curve was analysed to reveal two exponential components, one of $t_{0.5} = 2.5$ days and the other $t_{0.5} = 73$ days (see Fig. 2.4). These results indicated that the i.v. tracer dose of $^{65}$ZnCl$_2$ was eliminated from the body initially by a rapid process, and finally by a slow process.

2-5-2 Faecal excretion

The faeces were collected from the six animals used in the experiment for whole body counting. The $^{65}$Zn radioactivity in the faeces was assayed by gamma-ray spectrometry and the amount of $^{65}$Zn was calculated as a percentage of the injected $^{65}$Zn. The mean results are expressed graphically in Fig. 2.5.

It is clear that the i.v. trace dose was largely removed by faecal excretion. During the first 6 days approx. half the dose of $^{65}$Zn was recovered
Fig. 2.3. Radioactivity retained in the body of rats after i.v. injection of $^{65}$ZnCl$_2$. 
in the faeces, this was followed by a much slower excretion and more than 80% of $^{65}$Zn was excreted in 25 days. Larger individual variations between animals occurred in the slow excretion period.

![Graph](image)

**Fig. 2.4. Analysis of the whole body retention curve of $^{65}$Zn.**

**2-5-3 Urinary excretion**

The urine was collected from the six animals used in the experiment for whole body counting. The amount of $^{65}$Zn recovered in urine, expressed as a mean for the six animals, and calculated as a percentage of the injected dose of $^{65}$Zn is shown in Fig. 2.5.

The results clearly show that only small amounts of $^{65}$Zn was excreted in the urine. During the first 3 days after injection approx. 1% of the dose was excreted in urine and following this only trace amounts of $^{65}$Zn were detected in the daily collections. At least a part of this could be due to $^{65}$Zn contamination from faecal matter, hair and other exudates.
Fig. 2.5. Cumulative excretion of $^{65}$Zn in faeces and urine after i.v. $^{65}$ZnCl$_2$.

Points and bars are means ± s.e.m. for 6 rats, faeces (o) urine (o)
Fig. 2.6. Analysis of $^{65}$Zn clearance from the body.

Whole body $^{65}$Zn calculated as:

Whole body $^{65}$Zn = (100%) - (% cumulative faecal $^{65}$Zn)
The elimination of $^{65}\text{Zn}$ in the urine was not related to the volume of urine. However, for a given animal the ratio of daily faecal $^{65}\text{Zn}$ to daily urinary $^{65}\text{Zn}$ was approx. constant and was of the order of 100 for most rats.

Assuming that the faeces and urine were the only routes of excretion of $^{65}\text{Zn}$ from the animal, the $^{65}\text{Zn}$ retained in the body was calculated. The average results for the $^{65}\text{Zn}$ retained in the six rats are presented graphically in Fig. 2.6. Analysis of this curve revealed two exponential components, an initial rapid process ($t_{0.5} = 1.3$ days) and a slower process ($t_{0.5} = 14$ days).

2-5-4 The effect of additional oral ZnSO$_4$ on the excretion of $^{65}\text{Zn}$

In a group of four rats, two were force-fed with 1.0 ml of a solution containing 2.0 mg ZnSO$_4 \cdot 7\text{H}_2\text{O}$ in de-ionised water, by gastric intubation. The other two served as controls, and were given 1.0 ml of de-ionised water by intubation. Doses were given daily for 30 days, between 4.30 - 5 p.m. These animals had free access to water and solid food was offered in the night. During this period, no noticeable signs such as poor feeding, poor growth or any other symptoms due to intubation of ZnSO$_4$ were observed other than increased epilation in the ZnSO$_4$ treated rats. At the termination of the study the oesophagus and the stomach were opened and no visible damage was apparent. On the 31st day the
rats were injected i.v. with 20 μCi of $^{65}\text{ZnCl}_2$. Daily faecal and urine samples were collected from these animals for 7 days. This experiment was repeated twice more under identical conditions.

The radioactivity due to $^{65}\text{Zn}$ in faeces was calculated as a percentage of the injected $^{65}\text{Zn}$ dose. The ZnSO$_4$ group received 0.453 mg zinc ( = 2 mg ZnSO$_4$$\cdot$7H$_2$O ) daily, until the termination of the faecal collection. Both groups, the treated and controls received the normal dietary zinc. The amounts of $^{65}\text{Zn}$ recovered in the faeces are shown in Table 2-2.

Table 2-2 Faecal excretion of i.v. $^{65}\text{Zn}$ ($^{65}\text{ZnCl}_2$) after oral ZnSO$_4$

<table>
<thead>
<tr>
<th>Time after $^{65}\text{Zn}$ injection (days)</th>
<th>Cumulative faecal $^{65}\text{Zn}$ content (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZnSO$_4$ group</td>
</tr>
<tr>
<td></td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>1</td>
<td>12.59 ± 2.56</td>
</tr>
<tr>
<td>2</td>
<td>22.11 ± 2.87</td>
</tr>
<tr>
<td>3</td>
<td>26.02 ± 3.14</td>
</tr>
<tr>
<td>4</td>
<td>30.52 ± 3.28</td>
</tr>
<tr>
<td>5</td>
<td>33.56 ± 3.57</td>
</tr>
<tr>
<td>6</td>
<td>35.32 ± 3.92</td>
</tr>
<tr>
<td>7</td>
<td>37.18 ± 3.86</td>
</tr>
</tbody>
</table>
The results of the control group in this study compared favourably with the previous \(^{65}\text{Zn}\) study \(2-5-2\), for the cumulative faecal \(^{65}\text{Zn}\) content in the first seven days:

- Previous study: \(-\ 55.60 \pm 3.09\%\)
- Control group in this study: \(-\ 56.19 \pm 3.91\%\)

No significant difference was noted, \((P = 0.7)\).

However, the \(\text{ZnSO}_4\) treated rats showed decreased excretion of i.v. \(^{65}\text{Zn}\) (see table 2-2) and the difference shown by this small group of six rats was highly significant.

It thus appears that an increased intake of zinc orally resulted in the prolonged retention of an intravenously administered trace dose of \(^{65}\text{ZnCl}_2\).

The urine of the oral \(\text{ZnSO}_4\) treated animals did not show any appreciable difference in \(^{65}\text{Zn}\) content over the period of seven days:

- Control group: \(-\ 1.27\%\)
- \(\text{ZnSO}_4\) group: \(-\ 1.38\%\)

2-5-5  \(^{65}\text{Zn} \) excretion in pancreatic fluid and bile

Cannulated animals were injected i.v. with approx. 20 \(\mu\text{Ci}\) of \(^{65}\text{ZnCl}_2\) except for one animal given 40 \(\mu\text{Ci}\). The total amounts of \(^{65}\text{Zn}\) recovered in bile and the external secretions of the pancreas were estimated. Bile and bile and pancreatic fluid were collected hourly from a group of ten rats, five rats donating bile and the other five rats donating the combined secretions. \(^{65}\text{Zn}\) radioactivity was calculated as a percentage of the dose received by each animal. Results of this experiment are
shown in Table 2-3. The bile contained approx. 2% of the
dose of injected $^{65}$Zn, during 0-48 h, and approx. equal
amount of $^{65}$Zn was recovered each day. The combined
secretions contained 4-5 times more $^{65}$Zn in comparison
with the bile. More $^{65}$Zn was detected in the combined
secretions during the first 24 h, and the amount of $^{65}$Zn
secreted was reduced by approx. 50% over the next 24 h.
The results show that in the rat, at the dose levels
employed in this study, approx. 5 - 10% of $^{65}$Zn was
excreted in the pancreatic fluid.

$^{65}$Zn was detectable in both bile and pancreatic
fluid within the first hour after $^{65}$Zn administration.
The maximum pancreatic secretion of $^{65}$Zn occurred 2-4 h
after injection and then gradually diminished to a lower
level (Fig. 2.7). In contrast, $^{65}$Zn content of bile
gradually increased from an initial low level and
demonstrated a diurnal rhythm, Fig. 2.8. It is clear that
$^{65}$Zn is maximally excreted in bile during the day time
(morning) and the excretion diminished at night time. The
volume flow of bile is itself subject to a diurnal variation,
with a maximum flow around midnight and a minimum flow
around midday. Therefore more $^{65}$Zn is excreted per unit
volume of bile during the day than in the night. From
table 2-3 it can be seen that at higher dose levels the
bile and pancreatic secretions contained greater amounts
of $^{65}$Zn.
<table>
<thead>
<tr>
<th>Rat no:</th>
<th>Dose level (µCi/g b.wt.)</th>
<th>0 - 24 h</th>
<th>Bile and pancreatic fluid:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>65Zn(% dose)</td>
<td>Fluid vol.</td>
</tr>
<tr>
<td>1</td>
<td>0.0854</td>
<td>0.870</td>
<td>9.22</td>
</tr>
<tr>
<td>2</td>
<td>0.0866</td>
<td>0.992</td>
<td>10.79</td>
</tr>
<tr>
<td>3</td>
<td>0.0914</td>
<td>1.124</td>
<td>10.76</td>
</tr>
<tr>
<td>4</td>
<td>0.1107</td>
<td>1.463</td>
<td>9.78</td>
</tr>
<tr>
<td>5</td>
<td>0.1346</td>
<td>1.751</td>
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<tr>
<td>6</td>
<td>0.0797</td>
<td>1.650</td>
<td>26.00</td>
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<tr>
<td>7</td>
<td>0.0928</td>
<td>4.047</td>
<td>22.27</td>
</tr>
<tr>
<td>8</td>
<td>0.1174</td>
<td>6.721</td>
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<tr>
<td>9</td>
<td>0.1327</td>
<td>6.044</td>
<td>24.56</td>
</tr>
<tr>
<td>10</td>
<td>0.2125</td>
<td>7.711</td>
<td>28.17</td>
</tr>
</tbody>
</table>
Fig. 2.7. A typical $^{65}$Zn elimination in the external secretions of the pancreas and liver after i.v. $^{65}$ZnCl$_2$.

These results were obtained from two separate animals. Bile and pancreatic fluid (△) pure bile (○)

-64-
Fig. 2.8. $^{65}$Zn levels in bile after a single intravenous injection of $^{65}$ZnCl$_2$.

$^{65}$Zn ( o—o ), bile volume ( e—e ).

The night period is indicated by the horizontal dark bars. The arrows indicate 12 noon and midnight. The result shown was obtained in one animal.
Summary

In this study on the rat, the rate of elimination of $^{65}\text{ZnCl}_2$ from the body and the principal routes of excretion of $^{65}\text{Zn}$ were investigated using sub-physiological doses of high specific activity $^{65}\text{Zn}$. Results obtained from the counting of the whole body radioactivity indicated that the dose of $^{65}\text{Zn}$ was eliminated by two processes, with different rates, a fairly rapid process ($t_{0.5} = 2.5$ days) and a much slower process ($t_{0.5} = \text{approx. 73 days}$). The excretion of $^{65}\text{Zn}$ in faeces and urine accounted for the removal of most of the dose from the body, and after 25 days 80% of $^{65}\text{Zn}$ was excreted in the faeces and approx. 2% in the urine. Using the excretion data it was again shown that the elimination curve could be resolved into two components, one of $t_{0.5} = 1.3$ days and the other of $t_{0.5} = 14$ days. The increase of dietary zinc with orally administered $\text{ZnSO}_4$ produced a significant retardation in the faecal excretion of i.v. trace dose of $^{65}\text{ZnCl}_2$.

The experiment with cannulated rats provided information on the origin of the faecal $^{65}\text{Zn}$. During the first two days $1.5 - 2.8\%$ of the dose appeared in the bile and $6 - 10\%$ in the combined bile and pancreatic fluid. The $^{65}\text{Zn}$ content of the combined secretions accounted for $20 - 30\%$ of the faecal $^{65}\text{Zn}$ excreted in non-cannulated animals over two days.

The existence of a diurnal rhythm for $^{65}\text{Zn}$ excretion in bile was discovered, the bile samples collected around morning containing more $^{65}\text{Zn}$ than those collected around midnight.
CHAPTER 3

THE NATURE OF ZINC EXCRETED IN BILE

3-1 Introduction

The role of the liver in zinc metabolism remains obscure. In rats, this organ contains only 4% of the total zinc content of the body, but it localises about a third of a trace dose of radioactive Zn. A small proportion of $^{65}$Zn radioactivity is excreted from the liver daily in bile, and the excretion follows a diurnal rhythm. This suggests that the liver exercises some control over the biliary excretion of zinc and therefore it was decided to investigate the nature of $^{65}$Zn in bile in an attempt to obtain information on the possible mechanisms involved.

3-2 Experimental techniques

3-2-1 Animal experimentation

Male Wistar rats (approx. 220 g) were fitted with biliary cannulae as described in Section 2-2-3. Twenty four hours after cannulation the rats were injected with approx. 20 μCi of $^{65}$ZnCl$_2$, in the caudal vein. Bile samples were collected in bulk during the period of 0 - 24 h after $^{65}$ZnCl$_2$ injection, in a sterilised glass tube packed in ice. The bile was protected from light during collection by wrapping the glass tube with aluminium foil. Prior to use the glass tube was filled with an atmosphere of nitrogen obtained by pouring liquid nitrogen.
into the tube. Bile collected between 0-24 h was used for the investigations outlined in this Chapter. Bile collected in this way was a straw coloured fluid with a greenish tint and contained no solid matter when viewed under the light microscope at a magnification of x600. These rats secreted 6-8 ml of bile in twenty four hours and the pH of bile was 8.2 - 8.4.

3-2-2 Ultrafiltration

An ultrafiltration technique similar to that of Toribara et al. (1957) was used to investigate whether the $^{65}$Zn was bound to macromolecules in bile. The bile samples (3 ml), loaded into Visking dialysis tubing (Scientific Instrument Centre, London) cleaned in de-ionised water prior to use, were centrifuged in a specially constructed ultracentrifuge tube, Fig 3.1, at 1500 g for 30 min in an MSE 'minor' bench centrifuge to obtain an ultrafiltrate of approx. 0.3 ml. Accurately weighed amounts (approx. 0.1 ml) of the original sample and the ultrafiltrate were pipetted into counting vials and their radioactivity determined in a gamma-ray spectrometer. The binding of $^{65}$Zn to macromolecules was calculated using the following expression:

$$\% \text{ binding} = \frac{A - B}{A} \times 100$$

where,

$A$ = radioactivity of bile in c.p.s./g of bile

$B$ = radioactivity of the ultrafiltrate in c.p.s./g of ultrafiltrate.
Fig. 3.1 Ultracentrifuge tube containing the dialysis tubing loaded with sample.
Suitable controls were run to determine whether binding of $^{65}$Zn to the cellulose material of the Visking tubing was involved.

3-2-3 Exhaustive dialysis.

A dialysis tank was constructed for dialysing small quantities of fluid. The tank had dimensions of 16 x 13 x 12cm, was constructed out of perspex, and had no metallic parts. The Visking dialysis sac containing the bile sample was secured to the centre fitment as shown in Fig. 3.2, and rotated by means of an external motor. After its use the tank was cleaned by total immersion in EDTA, dil. HCl, dil. NaOH, and de-ionised water.

Visking dialysis tubing, flat width 1.0 cm of average pore size 24 Å was cleaned in de-ionised water prior to use. The sample was introduced into a sac made by tying one end of the tubing. A clean glass bead was introduced in order to stir the contents of the sac, before tying the open end. The contents of the dialysis sac were dialysed against 2 l of the appropriate solution, for varying lengths of time. The extent of dialysability of $^{65}$Zn was calculated as follows:

$$\text{% dialysability} = \frac{\text{c.p.s of dialysed bile}}{\text{c.p.s./g of bile before dialysis}} \times 100$$

Suitable controls were run to determine whether binding of $^{65}$Zn to the Visking tubing was involved.

* litres
3-2-4 Gel-permeation chromatography

The following materials were used:-

Chromatography column, 2.5 x 100 cm,
Sephadex G-200 gel (superfine)
Borate buffer containing 0.05M-H$_2$BO$_3$, 0.05M-KCl,
1.0M-NaCl adjusted to pH 8.0 with 0.05M-NaOH.

This buffer was chosen as it has been shown to be effective in the separation of biliary proteins by Nakayma and Miyake (1965). The glass column, flow adaptors and associated parts were thoroughly washed in weak Teepol solution. Possible metal contaminants were removed with 1.0% EDTA solution. These detergents were removed by repeated washing with de-ionised water. Sephadex gels were swollen in de-ionised water over a boiling water bath for 5 h, and then suspended in borate buffer. A gel column was made by pouring the slurry upto 80 cm mark, and secured in position with flow adaptors at both ends. The column was equilibrated with buffer solution for 24 h prior to use. A constant upward flow was maintained by hydrostatic pressure difference of 15 cm of water. The separations were carried out at ambient temperatures (18-20°) initially, and were repeated at low temperatures (5-7°), by the passage of cold water through the cooling jacket. The column was protected from light during chromatography. The flow rate of the column was 9-10 ml/h and the void volume determined with 2% (w/v) blue dextran was 150 ml.

A bile sample, 2.0 ml was applied at the bottom of the gel bed and chromatographed in an upward direction. The
Fig. 3.2. The dialysis tank, a single dialysis sac secured to the rotatable centre fitment is shown.
Eluates were collected in clean glass tubes in volumes of 4.5 ml. Aliquots of 1.0 ml were taken into counting vials for $^{65}$Zn assay. The remaining samples were read in a spectrophotometer (Unicam SP 500), at 280 and 460 nm, against a borate buffer blank. A bovine serum albumin (BSA) standard was run and its elution volume determined. A new gel column was used for each experiment.

3-2-5 Thin-layer gel filtration

This technique was used as an adjunct to column chromatography for more efficient separation of smaller amounts of proteins.

Sephadex G-75 gel, 3.75 g was swollen in 50 ml of borate buffer at pH 8.0, for two hours over a boiling water bath and was poured on a 20 x 40 cm glass plate, and drawn to a thickness of 0.04 cm. The plate was placed in a commercial TLG apparatus (Pharmacia G.B, Ltd.). The upper buffer reservoir was filled with 50 ml of borate buffer and connected to the gel bed by a 17.5 x 5 cm Whatman 3MM filter paper wick and the continuity to the receiving reservoir was made similarly. The gel bed was equilibrated at an angle of 20° overnight and the plate was lowered to a horizontal position for sample application. After application of 10 µl of the test sample the plate was inclined at 15° and the sample was allowed to migrate downwards. After four hours the plate was lowered and the buffer wicks disconnected. The plate was left in an oven at 40° to remove excess moisture and the proteins were visualised by:—
(a) Replica transfer method

A Whatman no. 1 filter paper 50 x 20 cm was unrolled along the length of the gel bed. The paper was removed from the gel and dried in an oven at 60° for 0.5 h. The proteins were fixed in a bath of 10% (w/v) trichloroacetic acid solution and stained with 1% (w/v) Ponceau S in 10% (v/v) acetic acid solution.

(b) N-chloro derivatives

After chromatography the plate was exposed to an atmosphere of chlorine vapour for 5 min. The excess chlorine was neutralised by spraying the plate with 20% ammonium sulphate and 5% sodium bicarbonate (w/v) in aq. solution. After 15 min, an aq. solution of 1% starch also containing 1% potassium iodide (w/v) was sprayed over the gel. Iodine formed by the N-chloro derivatives was visualised as blue spots. This method is more sensitive than (a) but the test is not specific for proteins.

3-2-6 Paper electrophoresis

Paper electrophoresis was carried out in a horizontal electrophoresis apparatus (Shandon Ltd., London). About 20 μl of the bile samples were applied as a thin band on Whatman 3MM paper (12 x 2.5 cm), 2 cm from the cathode wick. The buffer solution used was 0.1M-H₃BO₃ adjusted to pH 8.4 with 0.1M-NaOH. The bile samples were subjected to electrophoresis at 20 V/cm for 1.2, and 4 h periods at 4°. After electrophoresis the strips were dried in an oven at 40° in a free hanging position. The following spot tests were applied on individual strips:
(a) Proteins

Ponceau S reagent as described in Section 3-2-5.

(b) Amino acids

Papers were sprayed with 4% (w/v) ninhydrin soln. in acetone (1:19), and heated at 110° for 5 min.

(c) Bilirubin

Papers were sprayed with a freshly prepared diazo reagent. The reagent consisted of 96% ethanol (10ml) added to 0.1% aq. sulphanilic acid soln. (10ml) and 0.5% NaNO₂ soln. (0.6ml). Sulphanilic acid was diazotised in the sodium nitrite soln. in HCl, in the cold.

(d) Cholesterol

The papers were dipped in conc. H₂SO₄ - acetic anhydride (3:2) and flattened on a glass plate.

(e) Glucuronic acid

The papers were dipped in 0.2% napthoresorcinol in aq. H₃PO₃ (9%, v/v) and heated at 95° for 5 min.

(f) Bile acids

The papers were sprayed with 2% (w/v) vanillin in ethanol, then dipped in 85% aq. H₃PO₄ and heated to 70°.
(g) Lipoproteins

The papers were dipped for one hour in a saturated soln. of sudan black B in 55% ethanol and the surplus stain was removed by washing the papers with 40% ethanol.

3-2-7 Electro-immunodiffusion

An electrophoretic apparatus suitable for two dimensional electrophoresis was developed. The apparatus (Fig.3.3) was made out of perspex and consists of:-

(a) Buffer tank

This is a four compartmental vessel containing a platinum electrode in each compartment. The four power terminals are connected to a switch which is used to make or break the current flow and to direct the current across one pair of electrodes at a time.

(b) Cooling platen

This is used for supporting the Agarose gel plate during electrophoresis and can be cooled by cold water circulation. In these experiments the cavity of the platen was filled with cold water. In the first dimension electrophoretic run using 24 V/cm the gel bed tended to warm up and this was overcome by cooling the apparatus in a refrigerator before use and during electrophoresis. A series of four platens can be stacked vertically to support four gel plates, which allows four uniform separations to be obtained simultaneously.

-76-
Fig. 3.3. Two dimensional electro-immunodiffusion apparatus.
Buffer tank (A), Cooling platen (B), Lid of the cooling platen (L)
The position of an Agarose gel plate on B and the electrode wicks are shown.
A bed of Agarose gel was prepared by pouring 4.5 ml of a solution containing 3 ml of 2% (w/v) Agarose (Paines & Byrne Ltd., London) and 3 ml of 0.04M-barbitone-lactate buffer (pH 8.4), on a 5 x 5 cm clean glass plate. After the gel had set the excess was prised off leaving a 1 cm strip along one edge of the plate. Four ml of 1% Agarose gel prepared as before was mixed with 0.2 ml of the appropriate antiserum (see Section 3-2-9) at 40°. This was poured onto the plate to reconstitute the complete bed once again. A well was punched in the antiserum-free Agarose gel, half a centimeter from one corner on the diagonal line.

The glass plate containing the gel was placed on the cooling platen. Buffer wicks were cut out from Whatman 3MM filter paper and soaked in the barbitone-lactate buffer. Two layers of 1 cm wide wicks were used to connect the ends of the antiserum-free gel to the respective electrode compartment for the first dimension run, while 5 cm wide wicks were used for the electrical continuity of the second dimension run. The four compartments of the buffer tank were each filled with 250 ml 0.02M-barbitone-lactate buffer, pH 8.4 and the whole assembly was cooled to 4° one hour prior to loading the wells with 10 µl of protein solution for electrophoresis. Current was applied for 1 hr at 24 V/cm in the first dimension and for 8 h at 2V/cm in the second dimension.

After electro-immunodiffusion the excess antiserum was removed by washing the gel for 2 h in 0.9% NaCl adjusted to pH 8.8 with 1M-NaOH. The excess saline was in turn removed with short rinses in de-ionised water. The Agarose
was dried to a thin film in an oven at 60°. The preparations were then layered with 5 μ thick Melinex sheets (ICl Ltd) and autoradiographed using Kodirex film (Kodak Ltd). The immuno-precipitated proteins were visualised by dipping the Agarose plate in 1% (w/v) Ponceau S in 10% (w/v) acetic acid soln.

3-2-8 Immunelectrophoresis

The electrophoretic separation of samples investigated by this technique was carried out in the apparatus described in section 3-2-7, at 4°.

The Agarose gel was made by pouring 3.0 ml of a solution of 2% (w/v) Agarose in 0.03M-tris-barbitone sodium barbitone buffer of pH 8.8 (Gelman Instrument Co., Ann Arbour, Michigan, U.S.A), on a 2.5 x 7.5 cm glass plate. After the gel had set, wells (1mm diameter) and troughs (1 x 45 mm) were cut using a standard punch (Shandon Ltd., London). About 10 μl sample of the proteins to be separated was placed in the wells and electrophoresed in 0.3M-tris-barbitone sodium barbitone buffer for 0.75 h at 6 V/cm. After electrophoresis 0.4 ml of the appropriate antiserum (see Section 3-2-9) was placed in the trough and immuno-precipitation allowed to occur for 14 h at 37° in a moist chamber. The Agarose plate was washed to remove excess of antiserum, 65Zn was located by autoradiography and the proteins visualised with Ponceau S. (see Section 3-2-7).
3-2-9 Raising of antisera

Antisera for rat serum proteins and rat biliary proteins were obtained by immunisation of rabbits. Rabbits were chosen for the raising of antisera as their antisera show marked precipitation lines on the Agarose gel over a wide range of antisera concentrations. The method used was as described by Campbell et al. (1970).

Two male rabbits, Dutch strain (approx. 2.5 kg) were used. Blood serum was obtained from six normal healthy adult rats (approx. 200 g) and the pooled serum sample was thoroughly dispersed in Freund's complete adjuvant (Difco Laboratories, U.S.A) in a ratio of 4:1 (serum: adjuvant). The emulsion thus obtained was deposited in five subcutaneous sites in the nuchal region of rabbits and in each hind foot pad, in amounts of 0.1 ml per site. Two weeks later, 0.25 ml of fresh rat serum was injected into the ear vein to boost the immune reaction. The rabbits were bled 3 days later to check the antibody titer. Subsequently, when antisera were required the animals were boosted subcutaneously with serum in Freund's complete adjuvant, 7 days before bleeding.

For raising antisera to rat bile three rabbits weighing approx. 2 kg were used. Fresh rat bile samples obtained from three healthy rats (approx. 200 g) were pooled and thoroughly dispersed in an equal volume of Freund's complete adjuvant and this emulsion was deposited in volumes of 0.2 ml at five subcutaneous sites in the nuchal region and in each hind foot pad. Thereafter at fortnightly intervals 0.3 ml of fresh bile samples were injected intravenously into
the ear vein for three months. The i.v. dosage of bile in volumes between 0.6 - 0.75 ml produced discomfort, respiratory arrhythmia and temporary loss of gait, within a few minutes of injection, with recovery after about 30 min. If the i.v. dose was about 1.0 ml of bile, convulsions, severe body tremors and respiratory arrest leading to death was observed. A dose of 0.3 ml of bile produced no observable discomfort or any other toxic symptoms in single, or multiple dosage at fortnightly intervals. A final booster dose of bile in Freund's complete adjuvant was given subcutaneously seven days before collecting the antisera. The animals were bled 3 days after the first i.v. dose and after three months of the initial dose to check the antibody titer.

For the determination of the antibody titer, the passive haemagglutination method similar to that described by Campbell et al. (1970) was used. The agglutination of tanned human group-0 red blood cells coated with pure rat serum and pure bile, respectively, was checked using serial dilutions of the appropriate antisera. Both antigen and the antibody solutions were heated to 56°C for 30 min to inactivate the complement which was then absorbed with an equal volume of group-0 red blood cells.

A titer of 20,000 was obtained in one rabbit for rat serum protein antiserum. Further immunisation of this animal was delayed until seven days before the requirement of antiserum. The three rabbits immunised with bile gave a titer of 100 after the first i.v. dose and with further
immunisation a titer of 5000 was obtained in one rabbit after three months. The animals showed signs of hypersensitivity towards i.v. injections of bile at this stage.

Two animals from the respective groups with high titer antisera were selected as antisera donors, and when antisera were required these were bled from the ear vein and blood serum was obtained in the usual manner. These antisera were used for immuno electrophoresis and electro-immunodiffusion without further isolation or purification.
Materials

The glassware used for the collection of body-fluids and for the analytical techniques such as dialysis, chromatography, electrophoresis and ultrafiltration were cleansed by treating with the following solutions to decontaminate radioactivity from the previous experiments and to remove possible metal contaminants:

(a) 1% EDTA - soak for 24 h
(b) 10% Chromic acid - soak 24 h
(c) 5% NaOH - boil 2 h
(d) 10% HCl - boil 2 h
(e) De-ionised water - wash and rinse

Between each step the detergent left from the previous treatment was removed by rinsing in distilled water.

De-ionised water:

Glass distilled water was siphoned through a commercial deioniser, Elgastat (Elga Products, Bucks.), where the electrolyte content of the water was reduced by flow through an electrolyte cell and was further de-ionised by cation and anion exchange resins.

All the chemicals were obtained from the BDH Chemicals Ltd. The standard proteins for molecular weight determination were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.
3-4 Experimental results

3-4-1 Ultrafiltration of $^{65}$Zn in bile

Two studies were carried out, one at room temperature and the other at $4^\circ$ using bile samples from nine animals injected with 20 $\mu$Ci $^{65}$ZnCl$_2$ and two types of control solutions were used. The first solution contained 0.9% NaCl in de-ionised water and the second an ultrafiltrate of bile. The latter was obtained by centrifugation of 7 ml of bile in 24 $\mu$ pore size Visking tubing kept in large ultracentrifuge tubes (2.5 x 9.5 cm). These were centrifuged for one hour at $4^\circ$ to obtain an ultrafiltrate of 1 ml. The ultrafiltrate of bile has a straw green colour, probably due to bilirubin. Both saline and bile ultrafiltrate were separately mixed with 0.1 ml of $^{65}$ZnCl$_2$ to give counting rates similar to that of bile samples.

The pH of the resulting solutions was elevated from their normal pH of 6.4 and 7.8 to the pH of bile (8.3) by the addition of 0.1M-NaOH. Duplicate $^{65}$Zn added saline controls were centrifuged at the same time as bile samples. The $^{65}$Zn added bile ultrafiltrate was centrifuged in triplicate at room temperature and at $4^\circ$, on one occasion.

The ultrafiltrates of both control solutions contained $^{65}$Zn. The counting rates of these were compared with their original solutions and gave a recovery of 95 - 98% in the ultrafiltrate. The solution left in the dialysis bag also gave counting rates similar to that prior to ultrafiltration. This indicated the absence of $^{65}$Zn binding to the cellulose
material or to the glass walls of the ultracentrifuge tubes at this low $^{65}$Zn concentration. Therefore this method was used for the study with bile.

The $^{65}$Zn in bile samples did not filter through the 24A pores of the Visking tubing i.e., no $^{65}$Zn was detected in the ultrafiltrate of bile. This together with ultrafiltrability of $^{65}$Zn in $^{65}$Zn added to bile ultrafiltrate indicated the absence of $^{65}$Zn binding by ultrafiltrable low molecular weight materials in bile, under these conditions. Centrifuging samples at room temperature or at 4° had no effect on the extent of ultrafiltrability of $^{65}$Zn in bile or the controls. The results of this experiment is summarised in Table 3-1.

Table 3-1. Ultrafiltration of bile

<table>
<thead>
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<th>$^{65}$Zn in:</th>
<th>% binding at:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$18^\circ$</td>
<td>$4^\circ$</td>
<td>$18^\circ$</td>
</tr>
<tr>
<td></td>
<td>Mean ± s.d.</td>
<td>Mean ± s.d.</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Bile*</td>
<td>98.6 ± 3.46</td>
<td>96.3 ± 3.42</td>
<td>Bile ultrafiltrate**</td>
</tr>
<tr>
<td>Saline control*</td>
<td>6.44 ± 2.65</td>
<td>4.48 ± 2.01</td>
<td></td>
</tr>
</tbody>
</table>

* Six determinations at each temperature

** One determination in triplicate at each temperature
The dialysability of $^{65}$Zn in bile

Bile samples were obtained from nine rats injected (i.v.) with approx. 20 μCi of $^{65}$ZnCl$_2$. These samples were placed in 1.0ml amounts in Visking cellulose sacs and controls were prepared similarly, with 0.9% NaCl containing added $^{65}$ZnCl$_2$ of counting rates similar to that of test bile. Bile samples were dialysed against 2 l* of isotonic saline solution and the sacs were removed from the tank at 2, 4, 8, 24 and 48 h. The controls were dialysed similarly. The temperature of the dialysis system was maintained at 4° by leaving the apparatus in a refrigerator. This experiment was repeated at ambient temperatures, 18–20°.

In another experiment, bile samples obtained from three animals dosed with $^{65}$Zn were dialysed against 0.9% NaCl soln. which also contained $10^{-4}$mM-EDTA, and the pH of this solution was adjusted to 8.3 by the addition of 1.0M-NaOH. The temperature of the dialysis system was maintained at 4°. At the termination of dialysis gravimetrically known amounts of dialysed saline controls, dialysed bile and similar amounts of the original solutions of saline controls and bile were placed in counting vials and assayed for $^{65}$Zn radioactivity.

The results obtained in the experiments conducted at 4° are shown in Fig. 3.4. The $^{65}$Zn in the controls was readily dialysed; and after 24 h of dialysis complete removal of $^{65}$Zn was achieved. The dialysis membranes of the sacs were removed at 24 and 48 h and counted for $^{65}$Zn

* litres
Fig. 3.4. Exhaustive dialysis of $^{65}$Zn in bile.

e—o Bile dialysed against saline.

x—x Bile dialysed against $10^{-4}\text{M-EDTA}$.

.65 Zn added 0.9% NaCl soln. (=control) dialysed against saline.

Each point is a mean of six determinations.
radioactivity. These contained only traces of $^{65}$Zn and accounted for less than 5% of the $^{65}$Zn lost from the control solutions. It was concluded that the loss of $^{65}$Zn from the sac contents was due to dialysis and not due to $^{65}$Zn binding to the cellulose membrane.

$^{65}$Zn in the bile samples was not removed under similar conditions and the result indicated the absence of diffusible low molecular weight $^{65}$Zn binding molecules in bile. Dialysis of $^{65}$Zn-containing bile against saline solution containing $10^{-4}$ mM-EDTA removed about 40% of $^{65}$Zn during the first 24 h and 80% over 48 h. Thus, EDTA effects the removal of $^{65}$Zn from the bile at a slow, linear rate over the 48 h period.

Dialysis at ambient temperatures gave similar results for all solutions and the rate of dialysis of saline controls and bile against EDTA was not markedly affected. In all the above experiments the dialysate was not assayed for $^{65}$Zn.

3-4-3 Gel-permeation chromatography to reveal $^{65}$Zn binding components in bile

The bile samples investigated here were those obtained from the animals used in the previous studies. The suitability of the borate buffer system for $^{65}$Zn containing bile separation was checked by dialysis of this bile against the borate buffer in the exhaustive dialysis system described previously in section 3-4-2, and $^{65}$Zn was not dialysable. Two chromatographic separations were carried out at 18-20°C.
and another at 5-7°.

The column eluates were scanned at 280 nm for proteins and at 460 nm for biliary pigments, principally bilirubin, and also assayed for $^{65}$Zn content. The results of these experiments were very similar, and the result of one experiment is given in Fig. 3.5.

Small quantities of 280 nm u.v. absorbing materials were eluted prior to and after the BSA standard, and the major absorbance peak occurred almost at a volume corresponding to the total volume of the gel bed (400 ml). Approx. 95% of the $^{65}$Zn applied to the column was recovered in the eluates. Of this 22-26% $^{65}$Zn was recovered in the eluates containing the high mol.wt. material, i.e. 150 - 350 ml. The majority of $^{65}$Zn i.e. 74 - 78% was eluted with low mol. wt. material. Traces of bilirubin were detected in the high mol. wt. region but the majority of bilirubin appeared with low mol. wt. u.v. absorbing material. Column eluate was checked up to 800 ml (not shown in the Fig 3.5) and this region contained no $^{65}$Zn or light absorbing material.

In a separate experiment it was found that bilirubin interferes with the measurement of u.v. absorption of BSA solutions at 280 nm. This interference may be present in bile and therefore accurate quantitation of 280 nm absorbing materials may not be possible. However, the presence of 280 nm absorbing materials could be indicated at a protein concentration above 30 µg/ml in the presence of 70 µg/ml bilirubin. Protein determination by the method of Lowry (Lowry et al., 1951) on the column eluates of bile was not successful owing to precipitation.
Fig. 3.5 Fractionation on Sephadex G-200 of bile obtained from rats injected with $^{65}$ZnCl$_2$. u.v. absorption (---), $^{65}$Zn (——) and the bile pigment measured by light absorption at 454 nm (dotted areas) are shown. The elution volume of bovine serum albumin (BSA) is indicated by the arrow.
The combined bile and pancreatic fluid collected together for 24 h were chromatographed similarly. This experiment showed additional $^{65}\text{Zn}$ peaks eluted prior to the major $^{65}\text{Zn}$ peak in bile Fig. 3.6. These peaks were all associated with material absorbing u.v. at 280 nm and probably represent $^{65}\text{Zn}$ containing proteins in the pancreatic fluid. This figure also shows the presence of larger amounts of $^{65}\text{Zn}$ in pancreatic fluid in the first 24 h after dosing, in comparison with bile.

3-4-4 **Molecular weight determination of the major $^{65}\text{Zn}$ binding component in bile**

The presence of a low mol. wt. $^{65}\text{Zn}$ binding species in bile was identified by column chromatography and the mol. wt. of this component was investigated by thin-layer gel filtration.

Rat bile samples obtained from the animals used in the previous studies were divided into 0.5 ml aliquots in sterile glass tubes and stored at 4°. A new sample was taken for each gel filtration experiment.

Chromatography of 10 $\mu$l samples of bile resolved six spots on Sephadex G-75 gels, and a typical chromatogram is shown in Fig. 3.7. The two fast moving spots had a migration distance similar to that of albumin and gamma-globulin of blood serum. The remaining spots were unique.
Fig. 3.6. Fractionation on Sephadex G-200 of bile and pancreatic fluid obtained from 
65Zn-dosed rats. u.v. absorption (---), 65Zn (---).

Absorbance at 280 nm.
Fig. 3.7 A thin-layer chromatogram on Sephadex G-75 gel. Chromatographed rat bile, rat serum and proteins of known molecular weight are shown. The arrows indicate the direction of buffer flow.

The bile collected during the first 3-4 days after cannulation shows six major protein spots typically, (right) after 4 to 5 days only five spots can be detected (centre). In this latter sample the protein spot with an $R_f$ value similar to that of albumin is lost and the gamma-globulin spot becomes more prominent.
to bile. The spot with the lowest migration distance was the only spot that contained $^{65}$Zn, and this was found by $^{65}$Zn assay of the Sephadex gel which was scraped off the glass plate after chromatography of bile samples applied as a band. A similar migrating spot was faintly visible in chromatographed serum but in this experiment it was not possible to determine whether it was identical to the $^{65}$Zn binding component in bile. No other $^{65}$Zn binding material could be detected in bile by this method, unlike the column procedure, probably because of the small quantities used. A number of proteins (10 µg/ml) of known mol. wt. were used to obtain a standard plot relating mol. wt. to migration distance (Fig. 3.8). The mol. wt. of $^{65}$Zn binding component was obtained by reference to the standard curve and was in the region of 10,000 ± 2000.

Both replica transfer method and N-chloro derivatives enabled all the above spots to be visualised, but the transfer method outlined the spots only faintly.

3-4-5 Investigation of $^{65}$Zn binding protein in bile using immuno techniques

Approx. 10 µl of bile samples were subjected to immunoelectrophoresis and electro-immunodiffusion. Rabbit antisera for rat bile and serum proteins were used in separate electrophoretic plates and on occasions in the same immunoelectrophoretic plate in the respective troughs.
Fig. 3.8 The relationship between the inverse of the migration distance relative to thyroglobulin and log molecular weight.

The plot was obtained from the data of thin layer chromatography of standard proteins (shown above) on Sephadex G-75 superfine gel. R & E indicate the lower and upper mol. wt. limit of fractionation. The mol. wt. range of the $^{65}$Zn binding complex of bile is indicated (8000-10,000).
Their cross reaction was checked against rat bile and serum.

In rat bile, three immunoreacting proteins were demonstrated by immunoprecipitation of electrophoresed bile samples (see Fig. 3.9 & 3.10). The precipitin arc close to the point of application was probably a gammaglobulin. The second precipitin arc was identified as albumin (i.e. albumin-like) by comparison with an electrophoretic plate on which serum was crossreacted with antiserum to serum as well as bile. The third precipitin arc occurred only in bile and $^{65}$Zn was found in association with this protein. This was found by autoradiography of electro-immunodiffusion and immunoelectrophoretic plates (see Fig. 3.9). The autoradiographs due to $^{65}$Zn, which has poor film 'fog' properties, at these low levels of radioactivity were barely visible after 6 months of exposure and generous over-development of the film. However, a faint outline of biliary $^{65}$Zn binding protein and the absence of Ag images corresponding to albumin and the gamma-globulin was noted. No free $^{65}$Zn was detected under similar conditions, and pure $^{65}$ZnCl$_2$ remained at the point of application.

Of the two methods, the electro-immunodiffusion produced clear well defined protein peaks. The immunoelectrophoretic method was employed to check for the dissociation of $^{65}$Zn which may have occurred during the long, second electrophoretic run of 8 h in electroimmunodiffusion. However, in this experiment no dissociation was noted. It was noticed that the majority of pigment was associated with the biliary $^{65}$Zn binding protein and therefore
Fig.3.9 Immuno-reacting proteins of rat bile.

The proteins were separated by immunoelectrophoresis.

$B =$ bile, $S =$ serum, BAS = antiserum to rat bile,
SAS = antiserum to rat serum.

Fig. captions continued...
Biliary proteins and serum proteins immunoprecipitated with rabbit antiserum to rat bile. Three biliary proteins and two serum proteins were immunoprecipitated. The fast migrating, weakly immunoprecipitated protein was present only in bile. Two anode seeking spots were produced by bile in addition to the precipitin arcs and these were coincident with the front end (=anodic end) of the precipitin arc of the $^{65}$Zn binding protein and albumin-like protein, respectively. These spots were stained with Ponceau S and nigrosine, and were not removed when electrophoretic plates were washed with 0.9% NaCl soln. The biliary pigments also associated these spots but were removed with saline.

Biliary proteins and serum proteins immunoprecipitated with rabbit antiserum to rat serum. Two biliary proteins have electrophoretic and immunogenic properties similar to serum albumin and a gamma-globulin.

$^{65}$Zn binding protein of bile. The dotted areas contained $^{65}$Zn and the broken lines outline the precipitin arcs due to biliary proteins immunoprecipitated with rabbit antiserum to rat bile and serum, respectively. Note the reaction of identity produced by the homologous antibody. When $^{65}$ZnCl$_2$ was added to bile (1μCi/ml)

notes continued.....
and subjected to immunoelectrophoresis, the albumin band was also labelled.

Fig. 3.9.d. The effect of biliary drainage on the composition of biliary proteins.

Two bile samples obtained 24 h (upper) and 96 h (lower) after bile duct cannulation were subjected to immunoelectrophoresis. The continued biliary drainage lowered the $^{65}$Zn binding protein and the albumin-like protein but the gamma-globulin arc became more prominent. $^{65}$Zn binding was not studied under these conditions.
Fig. 3.10 Electro-immunodiffusion of bile.

B = bile, BAS = bile antiserum.

Ten microlitres of bile obtained from $^{65}$Zn dosed rats was placed in the well and electrophoresed in the 1st dimension (←). Electrophoretically separated proteins were then electrophoretically forced into the biliary antiserum bed (↑). Proteins were stained with Bonceau S. Note the presence of three immunoreacting proteins in bile. Dotted areas contained $^{65}$Zn located by autoradiography.
it is likely that the pigment exists in association with this protein.

In a further experiment the $^{65}$Zn binding protein in bile was separated on thin-layer Sephadex gel as described in Section 3-4-4 and the relevant band of Sephadex gel removed. The protein was eluted from the gel with a few drops of 0.9% NaCl by centrifugation in a modified Toribara tube, by placing the gel over the sintered glass disc for centrifugation. The resulting protein solution was investigated by immunoelectrophoresis using bile antiserum. A precipitin arc was produced identical to the $^{65}$Zn binding protein observed in whole bile samples. This confirmed that the $^{65}$Zn binding protein of mol. wt. approx, 10,000 was identical with the $^{65}$Zn immunoreacting protein.

3-4-6 Paper electrophoresis of $^{65}$Zn-protein-complex in bile

In the previous experiments it was found that bilirubin normally accompanied the $^{65}$Zn binding protein in bile. This simple experiment was designed to study further the presence of other biliary metabolites in association with $^{65}$Zn binding protein. The containing bile was electrophoretically separated on Whatman 3MM filter paper, and a number of compounds were identified by spot tests. The Whatman paper was selected as a supporting medium for electrophoresis because the Agarose gel and Sephadex gel were not stable to some of the spot reagents.

Typical electrochromograms showing the areas of colour obtained with the spot reagent are shown in Fig. 3.11 and
the results are given in Table 3-2. Samples electrophoresed for 1, 2 and 4 h did not show any variation of the constituents associated with $^{65}$Zn protein zone. Although simple paper electrophoresis may not have separated all the components of bile, it is indicated that the $^{65}$Zn binding protein may also be associated with amino acids, simple peptides, bilirubin and glucuronic acid, cholesterol and possibly other lipids.
Fig. 3.11. **Electrophoretically separated biliary components.**

Ten microlitres of bile was applied as a thin band and subjected to zone electrophoresis at 20V/cm for 1 h, and spot tests done to locate the compounds shown above. A number of compounds migrated with the fast moving protein band and the position of $^{65}$Zn (not shown) corresponded with the fast moving protein band.
<table>
<thead>
<tr>
<th>Test for:</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>Three bands were visible, one at the point of application, a second weak band in the central zone and a third of maximum migration towards the anode.</td>
</tr>
<tr>
<td>Lipoproteins</td>
<td>Two bands, one coincident with the fast moving protein band.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Two narrow bands, one with the fast moving protein.</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>One bilirubin band, coincident with the fast moving protein band.</td>
</tr>
<tr>
<td>Amino acids</td>
<td>Two bands, one at the point of application and a wide band extending from the fast moving protein band towards the anode.</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>A wide zone extending from the point of application to the fast moving protein band abutting both major protein zones.</td>
</tr>
<tr>
<td>Bile acids</td>
<td>A wide band between the major protein zones but not coincident with them.</td>
</tr>
<tr>
<td>$^{65}\text{Zn}$</td>
<td>One centimeter strips were counted for $^{65}\text{Zn}$ and the majority corresponded with fast moving protein band.</td>
</tr>
</tbody>
</table>
Summary

A group of rats weighing approx. 200g were cannulated for the collection of bile. These were injected with approx. 20 μCi of $^{65}$ZnCl$_2$ in the caudal vein. Bile samples obtained during the first 24 h after dosing were analysed by a number of methods to study the chemical nature of $^{65}$Zn in bile.

Bile samples were subjected to ultrafiltration and it was found that $^{65}$Zn in bile was not ultrafiltrable. $^{65}$ZnCl$_2$ added to 0.9% NaCl soln. and to an ultrafiltrate of pure bile was completely ultrafiltrable. This experiment indicated that all of the $^{65}$Zn in bile was bound to macromolecular components. Exhaustive dialysis of bile samples against 0.9% NaCl did not remove $^{65}$Zn from bile. However, the presence of EDTA in the dialysing medium effected the removal of $^{65}$Zn from bile at a slow linear rate, and about 80% of $^{65}$Zn was dialysed over 48 h. This evidence suggested that $^{65}$Zn was strongly bound to macromolecules of bile. In Sephadex (G-200) chromatography $^{65}$Zn in bile was eluted in association with 280 nm absorbing materials and the majority of $^{65}$Zn (approx. 80%) was associated with a biliary protein of mol. wt. 10,000. Antisera for rat biliary proteins and rat serum proteins were cross-reacted with biliary proteins and it was confirmed that the $^{65}$Zn binding protein was an immunoreacting protein which is present in bile, and not in blood serum. In paper electrophoresis of bile a number of biliary constituents were found to associate the $^{65}$Zn binding protein, thus
leading to the conclusion that $^{65}$Zn excreted in bile is bound to a macromolecular complex.
CHAPTER 4

TISSUE DISTRIBUTION OF $^{65}\text{Zn(ZnCl}_2\text{)}$

4-1 Introduction

A number of studies have been reported on the tissue distribution of $^{65}\text{Zn}$ and these were reviewed in Chapter 1. The laboratory rat has been selected in most of these studies as a model experimental animal. Although a vast amount of information has been obtained in this species these initial studies were carried out with high doses of zinc carrying the radioactive tracer.

$^{65}\text{Zn}$ with a low concentration of carrier zinc was used in this study to permit the investigation of tissue distribution of zinc at physiological levels. This study was carried out in three sections:

$^{65}\text{Zn}$ distribution in major organs and tissues,

$^{65}\text{Zn}$ distribution in the liver, the pancreas, the duodenum and the prostate gland at the cellular level,

$^{65}\text{Zn}$ distribution in subcellular organelles of the liver tissue.

Previous studies on the tissue distribution of $^{65}\text{ZnCl}_2$ indicate that, in soft tissues, $^{65}\text{Zn}$ does not equilibrate until 3-7 days following the intravenous administration of a single dose of $^{65}\text{ZnCl}_2$. This study was designed to examine the tissue distribution of $^{65}\text{ZnCl}_2$ in the 'pre-equilibrium' period before 3 days and then up to 10 days.
An autoradiographic investigation was carried out on some of the more important tissues in zinc metabolism, namely, duodenum, liver, pancreas and the prostate gland. The times chosen for this study were 2, 24 and 72 h after administration of $^{65}$ZnCl$_2$.

As the liver has been shown to play a central role in zinc metabolism, $^{65}$Zn distribution in subcellular organelles of liver was investigated to obtain further information on the behaviour of zinc in this tissue.
4-2 Experimental techniques

4-2-1 Gross tissue distribution

Wistar albino rats (approx. 230 g) were used, and the intravenous dosing of $^{65}$ZnCl$_2$ and counting of tissue samples were carried as described in Section 2-1-4 and 2-2-3. At selected times after dosing the animals were anaesthetised with ether and samples of blood (8 ml) taken from the heart into a heparinised syringe. The animals were then killed by cervical dislocation and the following tissues were removed as quickly as possible:

- Liver
- Heart
- Pancreas
- Lungs
- Stomach
- Spleen
- Duodenum
- Kidneys
- Prostate gland
- Testes
- Thigh muscle

**Blood.** Blood samples (approx. 2 ml) were centrifuged at 600 rev/min in a bench centrifuge for 5 - 8 min. The supernatant plasma was pipetted out and 1 g aliquots were taken for the assay of $^{65}$Zn. The formed elements of blood were washed free of plasma proteins using a quantity of 0.9% NaCl soln. equal to the volume of plasma removed. The cells were gently dispersed in the saline and centrifuged as before. The supernatant saline soln. was removed and the blood cells were washed twice more. Finally the packed cells were transferred to the counting vials.

**Liver.** For the assay of $^{65}$Zn in the liver, tissue samples (approx. 1 g) were removed from the centre of the right
anterior lobe. To establish whether the distribution of $^{65}\text{Zn}$ in the liver was uniform, the distribution of $^{65}\text{Zn}$ in the different lobes of the liver was examined by taking samples (0.3 - 0.5 g) from each of the five major liver lobes from a group of rats. In another group of rats the total $^{65}\text{Zn}$ content of the liver was estimated by weighing the the whole liver and assaying a representative sample.

Pancreas. As the pancreas in the rat is a diffuse tissue suspended in the omentum and its distinction from adipose tissue is difficult, a guide dissection was prepared to show the pancreatic tissue delineated from the associated tissue. A rat was intravenously injected with 0.1% eosin soln. and five minutes later was sacrificed and the pancreas removed from the associated viscera and placed in a bath of 1.0% potassium dichromate soln. The eosin stained the pancreatic tissue and the dichromate stained the adipose tissue. A thorough study of this preparation enabled the pancreas in $^{65}\text{Zn}$ dosed animals to be dissected. Pieces of pancreatic tissue lying in the duodenal loop (head), from the splenic end (tail) and from the mid-portion were removed for the assay of $^{65}\text{Zn}$.

Stomach and duodenum. These were opened up and their contents removed by washing under a jet of saline. The excess saline was removed by lightly blotting the tissue between filter paper.

Prostate gland. This gland was removed as a whole and cleaned free of adipose tissue.
Heart. Heart was opened to expose the chambers and cleaned with a jet of saline to remove blood. The excess saline was blotted off.

Other tissues. Lungs, spleen, the left kidney and the left testicle were removed from the carcass and assayed whole.

All the muscles of the left thigh were removed from the femur.

All tissues were transferred into preweighed counting vials as they were dissected out and the vials were capped to prevent the dessication of the tissues. When all the organs had been transferred, the bottles were reweighed and the $^{65}$Zn radioactivity assayed in the gamma-ray spectrometer as described in Section 2-2-3.

The tissue distribution of $^{65}$Zn was also studied in a group of rats which were given an additional oral intake of unlabelled ZnSO$_4$ as described in Section 2-4-5.

4-2-2 Autoradiography

A group of six rats were each injected with 20μCi of $^{65}$ZnCl$_2$ and killed at selected time intervals. Samples (approx. 3 mm cubes) were taken immediately from the median lobe of the liver, the tail of the pancreas, the left dorso-lateral lobe of the prostate gland and the duodenum near the opening of the bile duct.

The tissue samples were frozen by immersion in n-hexane cooled to $-70^\circ$ in a bath of solid CO$_2$ and acetone. The frozen tissue was removed from the n-hexane bath within a minute and placed on a pre-cooled microtome chuck which was kept in the cryostat (Slee, London), at $-30^\circ$. 

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Tissue was glued to the chuck with a thin film of Tissue Tek (Ames CO., U.S.A.) surrounding the tissue cube at its base. After 15 - 20 min the top layer of tissue cube was sliced off in 20μ sections to expose the tissue in the centre. This central tissue was least exposed to n-hexane, Tissue Tek, sudden temperature elevation from -70° to -30° and moist atmosphere of the cryostat. Histological sections (5μ thick) were cut and picked-up directly on AR.10 Stripping film for autoradiography (Kodak Ltd., London). The films were maintained between 0° to -4° for proper picking-up. Once a section was removed the cutting area of the knife was thoroughly cleaned with a moist tissue paper before the next section was cut.

The films were exposed in light-tight boxes containing silica gel for approx. 4 months at -10°. After exposure the preparation was allowed to warm to room temperature and the histological section fixed using 10% (v/v) formaldehyde solution for 10 min. Excess formaldehyde was removed by washing in distilled water. Photographic development was carried out as described by the manufacturer (Kodak Ltd.). Histological sections were stained with freshly diluted Ehrlich's haematoxin soln. (haematoxin:water; 1:3) and 1% aq. eosin. The preparations were dried in air and mounted on microscope slides using a drop of Euparal for each slide.

The autoradiographs thus prepared were examined under a light microscope at magnifications of x 95, x 240 and x 600 to study the Ag grain distribution in relation
to cell types. In some studies the grain density was estimated by counting the grains using a graticular eyepiece. This was done at a higher magnification, x 1000 using an oil immersion lens, and the field of view within the area (0.178 x 0.178 mm) of the graticule will be referred to as a unit area.

4-2-3 Subcellular distribution of $^{65}$Zn in the liver

Rats were injected with 20μCi of $^{65}$ZnCl$_2$ and killed by cervical dislocation at selected times after dosing. The livers were removed immediately and perfused through the hepatic vein with 0.23M-sucrose soln. chilled in ice. About 0.5 g of perfused liver was excised from the median lobe for $^{65}$Zn assay and the remainder was subjected to homogenisation and differential centrifugation.

The livers from two rats were combined and squeezed through an ice-cold stainless-steel mincer (pore size 0.1mm). The pulp was homogenised in 0.25M-sucrose (1:4 by weight), in a Potter Elvehjem homogeniser with 12 complete strokes. Preparation of the subcellular fractions was achieved using the differential centrifugation scheme outlined in Fig. 4.1.

All procedures were done between 0° and 4° using unbuffered 0.25M-sucrose soln. The final pellets were dispersed in 0.25M-sucrose with the aid of Pasteur pipettes followed by mixing in a whirly mixer. Samples of each homogenate were taken in duplicate for $^{65}$Zn assay (1 ml) and protein estimation (0.2 ml).

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Protein content of the samples was estimated using the procedure of Lowry et al. (1951). The whole homogenate was diluted 1:100, the lysosomal fraction 1:25 and all other fractions 1:50. A standard plot was prepared using crystalline BSA soln. in 1M-NaOH. The absorbance of the protein-reagent chromophore was read in a spectrophotometer (Unicam SP 500) at 750 nm.
Fig. 4.1. Scheme for preparation of subcellular fractions.

Homogenate (5 ml)
600g x 10 min*

Crude nuclear pellet¹ (3 ml)
50,000g x 1 h⁺

Supernatant with surface plug of whole cells, erythrocytes and mitochondria

Washed nuclear pellet² (5 ml)

Mitochondrial pellet² (3 ml)
4,000g x 10 min*

Supernatant

Supernatant

Lysosomal pellet and light mitochondria² (3 ml)
15,000g x 20 min*

Supernatant

Washed mitochondria² (5 ml)

Supernatant

Lysosomes and light mitochondria² (5 ml)

Supernatant

Cytosol

Supernatant

Microsomal pellet (5 ml)

(Notes next page)
Notes for Fig. 4.1.

The underlined fractions were assayed for $^{65}$Zn.

The volume in brackets indicate the volume of homogenate and suspended pellets equivalent to 1 g of original tissue.

* MSE High Speed (13000) centrifuge using 8 x 25,13° angle head rotor.

+ Spinco Ultracentrifuge (Model L) using 12 x 12, No. 40 rotor.

1 Resuspended in 2.4M-sucrose using two slow strokes in ground glass homogeniser with a loose-fitting pestle.

2 Resuspended in 0.25M-sucrose.
4-3 Materials

The autoradiographic film, Stripping film AR.10 was obtained from Kodak Ltd., London and the photographic chemicals from Johnson Ltd., London. Histochemicals were obtained from R.A.Lamb, London.
4-4 Experimental results

4-4-1 Tissue distribution

Male rats were injected intravenously with 20μCi $^{65}\text{ZnCl}_2$, killed at selected times and the tissues and blood assayed for $^{65}\text{Zn}$. The distribution of $^{65}\text{Zn}$ was studied over three different time periods:

- **Short term** - from 10 to 40 min after injection
- **Medium term** - from 1 to 6 h after injection
- **Long term** - from 1 to 10 days after injection.

The experimental design was:

**Short term**

<table>
<thead>
<tr>
<th>Time of killing (min)</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

**Medium term**

<table>
<thead>
<tr>
<th>Time of killing (h)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Long term**

<table>
<thead>
<tr>
<th>Time of killing (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The $^{65}\text{Zn}$ in the blood and tissues was expressed as a percentage of the injected dose per gram wet weight of tissue.

**Plasma.** The disappearance of $^{65}\text{Zn}$ from the plasma over the first 6 h is shown in Fig.4.2. Analysis of the plasma clearance curve indicated the presence of two components with half-lives of 0.85 h and 4.4 h, respectively. The experimental point
Fig. 4.2. The analysed plasma clearance curve.

For the evaluation of the slower process the clearance curve was considered up to 24 hours (not shown in the figure).

mean ± S.D. for 5 animals
determined for two hours was anomalous and was significantly different (p ≈ 0.01) from the anticipated point of the curve. $^{65}$Zn was still detectable in the plasma up to 10 days after injection and the long term values are given in Table 4-1.

Table 4-1 Long term plasma clearance of $^{65}$Zn

<table>
<thead>
<tr>
<th>Time of withdrawal of blood after $^{65}$Zn injection (days)</th>
<th>$^{65}$Zn in blood plasma (% of dose/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mean ± s.d</td>
</tr>
<tr>
<td>1</td>
<td>0.068 ± 0.007</td>
</tr>
<tr>
<td>2</td>
<td>0.029 ± 0.008</td>
</tr>
<tr>
<td>4</td>
<td>0.012 ± 0.008</td>
</tr>
<tr>
<td>5</td>
<td>0.017 ± 0.003</td>
</tr>
<tr>
<td>6</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td>9</td>
<td>0.012 ± 0.003</td>
</tr>
<tr>
<td>10</td>
<td>0.006 ± 0.002</td>
</tr>
</tbody>
</table>

Values are means ± s.d. for three rats.

Liver The concentration of $^{65}$Zn in the different lobes of the liver was investigated in a group of 8 rats, intravenously injected with 20μCi of $^{65}$ZnCl$_2$. Five rats were killed 2 h after the injection and the others after 24 h, and the $^{65}$Zn assays were carried out on liver samples taken from the five lobes. $^{65}$Zn assay results are shown in Table 4-2. These results indicated fairly similar $^{65}$Zn concentrations in the five lobes. Therefore a tissue sample from any one lobe could be assayed to estimate the
In another experiment a group of rats were injected (i.v.) with 20μCi of $^{65}$ZnCl₂. The livers were removed at 0.33, 2, 6, 24 and 48 h after injection and weighed whole. A sample (0.5 g) was taken from the anterior right lobe for $^{65}$Zn assay. $^{65}$Zn content of the whole livers are shown in Table 4-3. It can be seen that more than a third of the tracer dose entered the liver initially but the amount fell rapidly to approx. 15% at 6 h. From 6 to 48 h $^{65}$Zn content in the liver fell slowly.

In another experiment, the uptake and clearance of $^{65}$Zn in the liver was examined on a wider time scale from 10 min to 10 days. Liver samples were obtained from rats used in the gross tissue distribution studies, where other tissue samples were analysed too. These rats were dosed normally...
and killed at selected times after dosing. $^{65}$Zn assays were performed on liver samples obtained from the right anterior lobe (0.5 g) and $^{65}$Zn concentrations expressed as a percentage of the injected dose. The results are given in Tables 1 and 2 given in the Appendix.

The $^{65}$Zn concentration curve of the liver is presented in Fig. 4.3. The curve shows an uptake portion and a clearance portion demarcated by a peak concentration level. The maximum $^{65}$Zn concentrations were achieved rather rapidly, i.e., before one hour. Once the peak concentrations were reached $^{65}$Zn was at first released from the liver rather rapidly, but the clearance rate was slower from 6 h to 3 days, and became extremely slow from 4 to 10 days.

Table 4-3 $^{65}$Zn concentration in the whole liver

<table>
<thead>
<tr>
<th>Time after injection (hours)</th>
<th>Liver $^{65}$Zn content (total % of dose)</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.d.</td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>40.27 ± 3.22</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>22.79 ± 4.13</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>15.67 ± 3.48</td>
<td>4</td>
</tr>
<tr>
<td>24</td>
<td>15.58 ± 2.67</td>
<td>3</td>
</tr>
<tr>
<td>48</td>
<td>13.78 ± 2.82</td>
<td>3</td>
</tr>
</tbody>
</table>

Pancreas Small tissue samples from the pancreas of the $^{65}$Zn dosed rats were obtained from the head, mid-portion and tail of the pancreas and were pooled for $^{65}$Zn assay.

-122÷
Fig. 4.3. \(^{65}\text{Zn}\) concentration in the rat liver after a single i.v. dose of \(^{65}\text{ZnCl}_2\).

Points and bars are means ± s.d.
In the pancreas, the concentration of $^{65}\text{Zn}$ increased up to 2 h, to reach a peak level (Fig. 4.4). From 6 - 72 h the $^{65}\text{Zn}$ concentrations decreased at a rate higher than the period following this, i.e. 3 - 10 days. Using these assay values the total loss of $^{65}\text{Zn}$ from the pancreas was calculated, and this amounted to 2 - $3\%$ of the dose for the period from 2 - 24 h and less, 1% from 24 - 48 hours.

Fig. 4.4 $^{65}\text{Zn}$ concentration in the rat pancreas after a single i.v. dose of ZnCl$_2$

Points and bars are means ± s.d.
Kidney The $^{65}\text{Zn}$ in the left kidney was assayed and the $^{65}\text{Zn}$ concentration, calculated as a percentage of the injected dose, is presented graphically (see Fig. 4.5). The kidney showed a rapid uptake of $^{65}\text{Zn}$ earlier than two hours and showed a gradual clearance through the course of the study.

\begin{figure}[h]
\centering
\includegraphics{fig4.5.png}
\caption{$^{65}\text{Zn}$ concentrations in the kidney following a single i.v. dose of $^{65}\text{Zn Cl}_2$. Points and bars are means $\pm$ s.d.}
\end{figure}
Duodenum A portion of the duodenum was removed from the $^{65}\text{Zn}$ dosed rats and the $^{65}\text{Zn}$ content expressed as a percentage of the injected dose as shown in Fig. 4.6. The duodenum showed fluctuating $^{65}\text{Zn}$ levels during the first 6 h after injection with maximum concentrations achieved around 2 h. The clearance of $^{65}\text{Zn}$ during 1-10 days occurred rather slowly, in a manner similar to that of the liver, pancreas and kidney.

Fig. 4.6. $^{65}\text{Zn}$ concentrations in the duodenum after a single i.v. dose of $^{65}\text{ZnCl}_2$.

Points and bars are mean ± s.d.
Fig. 4.7. 65Zn concentrations in the spleen, stomach, lung, cardiac muscle and voluntary muscle after a single intravenous injection of 65ZnCl2.

The points are mean values obtained in small groups of rats.
Time after injection

Fig. 4.8. $^{65}$Zn concentration in the prostate gland, testes and the formed elements of blood after a single intravenous injection of $^{65}$ZnCl$_2$. The points are mean values obtained in small groups of rats.
$^{65}$Zn concentrations in the liver, pancreas and kidney showed variations among the individual animals during the period 10 min to 6 h. From 1 to 10 days these tissues and particularly the duodenum, throughout the course of the study, showed smaller individual variations in $^{65}$Zn levels.

The spleen, lungs, stomach, heart and thigh muscle were obtained from the same animals and the $^{65}$Zn levels are expressed as a percentage of the injected dose as shown in Fig. 4.7.

**Spleen** $^{65}$Zn concentrations in this organ increased at a slower rate and then showed a slow clearance.

**Stomach** $^{65}$Zn concentration in the stomach remained almost steady during 10 min to 4 - 5 days, and the clearance was extremely slow.

**Lungs** The lungs showed an initial clearance of $^{65}$Zn up to one hour followed by a slow increase up to 24 h which then decreased slowly.

**Heart** Heart contained lower $^{65}$Zn levels than the lungs but otherwise behaved similarly to lungs in $^{65}$Zn uptake and clearance.

**Thigh muscle** Muscle showed slowly increasing $^{65}$Zn levels over the 10 days following injection.

$^{65}$Zn was assayed in the prostate gland, left testicle and blood cells of the same animals used for the other tissue assays and the results are shown in Fig. 4.8.

**Prostate gland** This showed an increase in $^{65}$Zn up to 24 h
followed by a slow clearance.

**Blood cells** These showed an initial decrease followed by an approx. constant level of $^{65}$Zn over 10 days.

**Testes** Testes slowly accumulated $^{65}$Zn for at least 6 days and showed a decrease afterwards.

The effect of additional oral ZnSO$_4$ on the tissue distribution of i.v. $^{65}$ZnCl$_2$.

A group of six rats were force-fed with 2.0 mg of non-radioactive ZnSO$_4$·7H$_2$O daily for 30 days. Three control rats were given de-ionised water by gastric intubation. On the 31st day all rats were injected with 20$\mu$Ci of $^{65}$ZnCl$_2$, intravenously. Twenty four hours later these were killed and $^{65}$Zn levels in plasma and tissues were assayed. The results are presented in Table 4-4. Daily ZnSO$_4$ feeding resulted in increased $^{65}$Zn levels in the kidney, liver, muscle, pancreas and duodenum while significantly lower levels were found in the spleen and the prostate gland. Plasma and stomach contained similar $^{65}$Zn levels to that of controls.

**4-4-2 $^{65}$Zn localisation in pancreas, liver, duodenum and prostate gland**

Six rats were injected with 20$\mu$Ci of $^{65}$ZnCl$_2$ in the caudal vein and killed in groups of two at 2, 24 and 48 h after injection. Histological sections of the liver, pancreas, duodenum and prostate gland were autoradiographed. Identical tissues obtained from a control rat demonstrated that chemical fogging was not
involved and the grain count obtained with these tissues were considered as the background levels.

The autoradiographs of the pancreatic tissues were examined at a magnification of x 600. At the three time intervals, 2, 24 and 48 h, Ag grains were located over the thin connective tissue capsule that surrounds the pancreas and over the interlobular septa that demarcate each lobule. In contrast, the tissue enclosed within the connective tissue capsule contained more Ag grains. Grains were counted over the islets of Langerhans (endocrine tissue) and over the acinar tissue (exocrine tissue) at a magnification of x 1000. Grain counts were made over the control autoradiographs similarly. The results of this experiment are shown in Table 4-5.
Table 4-4 \( ^{65}\text{Zn} \) tissue concentration in oral \( \text{ZnSO}_4 \), treated rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>( ^{65}\text{Zn} ) (% of injected dose)</th>
<th>( ^{65}\text{ZnSO}_4 ) _group</th>
<th>Controls</th>
<th>P value (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.d.</td>
<td>Mean ± s.d.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.038 ± 0.009</td>
<td>0.045 ± 0.007</td>
<td></td>
<td>(&lt; 0.25</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.027 ± 0.18</td>
<td>0.18 ± 0.027</td>
<td></td>
<td>(&lt; 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.56 ± 0.024</td>
<td>1.55 ± 0.032</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.06 ± 0.146</td>
<td>0.58 ± 0.097</td>
<td></td>
<td>( 0.2 &gt; P &gt; 0.1</td>
</tr>
<tr>
<td>Duodenum</td>
<td>2.25 ± 0.472</td>
<td>1.48 ± 0.109</td>
<td></td>
<td>(&lt; 0.05</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.17 ± 0.029</td>
<td>0.44 ± 0.036</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.53 ± 0.234</td>
<td>1.44 ± 0.133</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Liver</td>
<td>3.64 ± 0.533</td>
<td>1.98 ± 0.286</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.84 ± 0.268</td>
<td>1.22 ± 0.072</td>
<td></td>
<td>(&lt; 0.01</td>
</tr>
</tbody>
</table>

No. of rats 6 3

\( ^{65}\text{Zn} \) in tissues assayed 24 h after injection of \( ^{65}\text{ZnCl}_2 \)

* Result highly significant
Fig. 4.9. $^{65}$Zn localisation in the basal region of acinar cells of rat pancreas.

C = centroacinar cell, D = lumen of the acinus.

A diagrammatic representation of a pancreatic acinus. The pancreas was obtained 2 h after i.v. $^{65}$ZnCl$_2$ injection and a $5\mu$m thick histological section was autoradiographed. Black dots represent Ag grains due to $^{65}$Zn.

(viewed at a magnification of x 600 )
Fig. 4.10. $^{65}$Zn in the granular cytoplasm of acinar cells of rat pancreas.

A = acinar cell, N = nucleus

A diagrammatic representation of an acinus.

The pancreatic tissue was obtained 24 h after i.v. $^{65}$ZnCl$_2$ injection. Black dots represent Ag grain pattern in acinar cell autoradiographs. (viewed at a magnification of x 600)
Table 4-5 Silver grain counts in the autoradiographs of Pancreas

<table>
<thead>
<tr>
<th>Time after injection (hours)</th>
<th>Islet tissue</th>
<th>Acinar tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± s.d.</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>2</td>
<td>68 ± 12 (27)</td>
<td>77 ± 14 (27)</td>
</tr>
<tr>
<td>24</td>
<td>38 ± 8 (32)</td>
<td>35 ± 12 (32)</td>
</tr>
<tr>
<td>48</td>
<td>24 ± 8 (35)</td>
<td>20 ± 8 (35)</td>
</tr>
<tr>
<td>Control</td>
<td>4 ± 2 (9)</td>
<td>3 ± 2 (9)</td>
</tr>
</tbody>
</table>

Total number of areas counted are shown in brackets.

These results indicated a lowering of the grain count over the islets as well as the exocrine tissues from 2 to 48 h. The islet and acinar tissues contained similar grain densities at a given time interval.

A difference was noticed in the manner in which Ag grains were distributed within the component cells of the endocrine and exocrine tissue. In the exocrine tissue 2 h after $^{65}$Zn injection, a large number of acini frequently showed $^{65}$Zn localisation in the cytoplasm in the basal portion of the acinar cell (Fig. 4.9) and at 24 h most of the $^{65}$Zn was found to cluster in the central and apical areas of acinar cells in all acini (Fig. 4.10). The distribution of $^{65}$Zn in the islet tissue appeared uniform and no specific localisation within the cell could be
observed.

Liver The autoradiographs prepared from liver sections were examined at x 600 magnification, and numerous silver grains were found in the blood vessels, the cells and the sinusoids. At the time intervals of 2, 24 and 48 h, $^{65}\text{Zn}$ appeared to be uniformly distributed in the whole histological section of the liver.

Duodenum A small portion of the duodenum proximal to the opening of the bile duct was obtained 2, 24 and 48 h after $^{65}\text{Zn}$ injection. Autoradiographs of tissue sections revealed the presence of $^{65}\text{Zn}$ in all types of cell at all three time intervals. Autoradiographs obtained 2 h after $^{65}\text{Zn}$ injection showed that the tissues between the serosa and muscularis mucosa contained a smaller number of grains in comparison with the tissue between the muscularis mucosa and the duodenal lumen. Clusters of grains were visible at the base of crypts of Lieberkühn near the muscularis mucosa (Fig. 4.11 and 4.12). The end of the crypt near the lumen of the duodenum was less radioactive. Also the crypts cut transversely at a point above the basal region did not show the $^{65}\text{Zn}$ concentration noticeable in the basal portion of the longitudinally cut crypts confirming that the $^{65}\text{Zn}$ localisation occurred in the basal region of the crypts and not along its length. The villi at 2 h showed a uniform grain distribution and contained a lower grain density than the basal crypts. The localisation of $^{65}\text{Zn}$ seen in the basal crypts at 2 h had disappeared by 24 h. At 24 and 48 h more grains in the form of streaks running
Fig. 4.11. $^{65}$Zn localisation in the basal region of the crypts of Lieberkühn of rat duodenum. 
C = crypt, G = goblet cells, L = lumen of crypt, SM = submucosa

The arrows show an area with excess silver grains. The duodenum was obtained 2 hours after $^{65}$ZnCl$_2$ injection. (magnification x400)
Fig. 4.12. $^{65}\text{Zn}$ localisation in the Paneth cells of rat duodenum.

C = crypt of Lieberkühn, MM = muscularis mucosa, L = lumen of crypt, P = Paneth cell

High power photomicrograph of an autoradiograph of duodenum 2h after $^{65}\text{ZnCl}_2$ injection. Two crypts cut longitudinally are showing $^{65}\text{Zn}$ in Paneth cells and lumen of crypt. (magnification x1000)
Fig. 4.13. 65Zn localisation in the duodenal villi of the rat.

Autoradiographic visualisation of 65Zn localisation in the duodenum 24h after i.v. 65ZnCl₂. Arrows show two areas along the lumen showing 65Zn.

Note 65Zn at the lumen end of the goblet cells. (Magnification x600)
Fig. 1.13b. $^{65}\text{Zn}$ localisation in the duodenal villi of the rat.

Note $^{65}\text{Zn}$ localisation within lacteals (L) and goblet cells (G) 48 h after $^{65}\text{ZnCl}_2$ injection. (magnification x600)
Fig. 4.14. $^{65}\text{Zn}$ in rat prostate gland.

SU = secretory unit, S = stroma

An autoradiograph of a histological section of the dorso-lateral prostate gland taken 48 h after $^{65}\text{ZnCl}_2$ injection. $^{65}\text{Zn}$ associated with two secretory units can be seen. (magnification x240)
Fig.4.15. \(^{65}\text{Zn}\) secretion in the rat prostate gland.

S = stroma  \hspace{1cm} E = follicular epithelium, L = lumen

Autoradiograph shows \(^{65}\text{Zn}\) localisation in the luminal border of a secretory unit 24 h after i.v. \(^{65}\text{ZnCl}_2\). Note the manner in which \(^{65}\text{Zn}\) is secreted at localised spots along the epithelium. (magnification x600)
Fig. 4.16. $^{65}\text{Zn}$ labelled prostatic concretions.

L = follicular lumen, PC = prostatic concretion

Autoradiograph of rat dorso-lateral prostate gland 72 h after $^{65}\text{ZnCl}_2$ injection

The radioactivity near the epithelium is probably due to prostatic secretions.

(magnification x240)
in a longitudinal section, were localised over the centre of the villi in the lamina propria (Fig. 4.13a). At this time, localised clusters of silver grains were also noticeable in the duodenal lumen, in the spaces between the villi. These clusters were more concentrated near the goblet cells, and some goblet cells showed the presence of clusters of Ag grains within them (Fig. 4.13b) and at the luminal end of the cells.

**Prostate** Thin histological sections of the dorso-lateral prostate gland were autoradiographed to study the $^{65}$Zn distribution, 2, 24 and 48 h after injection of $^{65}$Zn. These autoradiographs showed the presence of $^{65}$Zn in the connective tissue capsule at the periphery of the gland, the fibromuscular stroma consisting of connective tissue and smooth muscle and the interstitial tissue with capillaries. These structures contained fewer Ag grains in comparison with the glandular follicles, some of which showed massive concentration of $^{65}$Zn within the follicular lumen (Fig. 4.14).

The epithelial cells lining the glandular cavity were heavily labelled particularly at 24 h (Fig. 4.15). Often, the secretion and concretions of the secretion also contained $^{65}$Zn in high amounts at all times studied (Fig. 4.16). The autoradiographs showed marked differences in grain density in different areas of the same section with some follicles containing a large number of grains and others in their immediate neighbourhood containing only a few grains.
Sixteen rats were injected intravenously with 20μCi $^{65}$ZnCl$_2$. These were killed in groups of four at 0.5, 2, 24, and 48 h after dosing, the livers were homogenised and subcellular fractions prepared.

The $^{65}$Zn content in 1.0 g of unhomogenised liver was compared with that in 1.0g of 100% whole homogenate. The results were as follows:

<table>
<thead>
<tr>
<th>Time after $^{65}$Zn injection (h)</th>
<th>Homogenate $^{65}$Zn x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean + s.d.</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>114 ± 4.24</td>
</tr>
<tr>
<td>2.0</td>
<td>107 6.82</td>
</tr>
<tr>
<td>24.0</td>
<td>98.7 4.44</td>
</tr>
<tr>
<td>48.0</td>
<td>92.5 4.82</td>
</tr>
</tbody>
</table>

Initially more $^{65}$Zn was recovered in the whole homogenate in comparison with the unhomogenised liver and the figures progressively decreased with time. This difference may be due to $^{65}$Zn in the connective tissue which did not pass through the mincer. In view of this all subsequent results have been related to the $^{65}$Zn content of the whole homogenate.

$^{65}$Zn content in the subcellular fractions was calculated as a percentage of the $^{65}$Zn content in the total homogenate and the results are shown in Fig. 4.17. The majority of $^{65}$Zn was present in the cytosol and decreased in order in
Fig. 4.17 \(^{65}\)Zn distribution in subcellular fractions of rat liver.

Subcellular fractions were prepared 0.5, 2, 24 and 48 h after i.v. \(^{65}\)ZnCl\(_2\) injection. The results shown for \(^{65}\)Zn content are mean values of 4 samples. The protein values are mean ± s.d. for nine samples. The bars for each subcellular fraction represent \(^{65}\)Zn values at 0.5, 2, 24 and 48 h respectively. S104 = cytosol (Lysosomal fraction is not shown).
Fig. 4.18. Fractionation on Sephadex G-200 of rat liver cytosol prepared from rats injected with $^{65}\text{ZnCl}_2$. u.v absorption (-o-), $^{65}\text{Zn}$ (-x-) and the elution volume of BSA and biliary $^{65}\text{Zn}$ protein are shown. Cytosol was prepared 24 h after $^{65}\text{ZnCl}_2$ dosing.
the microsomal, nuclear, mitochondrial and lysosomal fractions. The lysosomal fraction (not shown in the figure) contained the smallest amount of $^{65}\text{Zn}$ (i.e. 1-2%). Although in this study the microsomal fraction contained similar amounts of protein to that of nuclear and mitochondrial fractions the former contained more $^{65}\text{Zn}$ than the latter two fractions. The $^{65}\text{Zn}$ recovered in the subcellular fractions amounted to 91 - 95% of total homogenate content at the time intervals studied. The protein recovery was approx 95% in this experiment.

In the tissue distribution study it was seen that after $^{65}\text{Zn}$ injection, the $^{65}\text{Zn}$ content of the liver decreased from 0.5 to 48 h. This decreasing radioactivity was also shown by the total homogenate and the subcellular fractions except the microsomal fraction in this study. In contrast the microsomal fraction showed an increase in the $^{65}\text{Zn}$ content around 2 h, which then decreased after 24 h.

Samples of the cytosol obtained at 0.5, 2, 24 and 48 h were chromatographed on Sephadex G-200 under identical conditions to those described for bile (see section 3-2-4). The eluate was monitored at 280 nm for protein content and assayed for $^{65}\text{Zn}$. All samples showed an essentially similar elution pattern and the results for the 24 h samples are shown in Fig. 4.18. Approx. 95% of the radioactivity was recovered from the column in association with high mol. wt. material. The majority of the $^{65}\text{Zn}$ was associated with material of mol. wt. less than albumin but greater than 10,000. However, it was not possible to correlate with any discrete 280 nm absorption peaks.
In the region in which the $^{65}$Zn biliary complex was eluted in the study on bile, i.e. at an elution volume of 420 ml, approx. 2% of the $^{65}$Zn in the cytosol was eluted as a distinct peak. A smaller peak of $^{65}$Zn was noted at 440 ml.
The tissue distribution of $^{65}$Zn was investigated on a time-course basis after intravenous injection of $^{65}$ZnCl$_2$ in rats. Following the dosing $^{65}$Zn was detectable in blood plasma and all soft tissues assayed for $^{65}$Zn for a period of 10 days. The $^{65}$Zn concentrations occurred in decreasing order in the pancreas, liver, kidney, duodenum, spleen, stomach, lung, heart, prostate gland, testes, thigh muscle and the formed elements of blood, during the first 24 h after $^{65}$Zn injection. Each tissue had a characteristic $^{65}$Zn concentration curve and those for the liver and pancreas in particular showed an uptake phase and a clearance phase demarcated by a peak concentration level. The $^{65}$Zn uptake in the kidneys, liver, pancreas and duodenum was rapid in comparison with the spleen, stomach, lung, and heart and the prostate gland. However, after 1-2 days the $^{65}$Zn levels in all of these tissues decreased and at 10 days they all contained approx. the same amount of $^{65}$Zn per gram wet weight. The testes, voluntary muscle and the formed elements of blood slowly accumulated $^{65}$Zn. This study revealed that each of the soft tissues had a characteristic turnover of $^{65}$Zn. Additional oral ZnSO$_4$ feeding resulted in changes in the normal tissue distribution of a trace dose of $^{65}$Zn.

$^{65}$Zn distribution in the pancreas, liver, duodenum and prostate gland was investigated by autoradiography. In the pancreas, both exocrine and endocrine tissue contained
\[ ^{65}\text{Zn}, 2-48 \text{ h after injection.} \] The acinar cells showed higher levels of \(^{65}\text{Zn}\) in the cytoplasm at the base of the cells, 2 h after \(^{65}\text{Zn}\) injection and at 24 h and 48 h more \(^{65}\text{Zn}\) was localised in the cytoplasm in the central and apical areas. In the duodenum \(^{65}\text{Zn}\) was detected in the Paneth cells of the crypts of Lieberkühn and in the lumen of the crypts, 2 h after dosing. At 24 and 48 h \(^{65}\text{Zn}\) was localised along the centre of the lamina propria of villi, in the goblet cells and in duodenal lumen near the goblet cells. In the dorso-lateral prostate of sexually mature rats there was a great variation in the \(^{65}\text{Zn}\) distribution in glandular follicles with some showing a heavy \(^{65}\text{Zn}\) localisation within the epithelial cells of the secretory units and follicular lumen. In the liver tissue \(^{65}\text{Zn}\) was uniformly distributed in polygonal cells and Kupffer cells.

\(^{65}\text{Zn}\) distribution in the subcellular fractions of the liver of \(^{65}\text{Zn}\) dosed rats was investigated. All subcellular fractions contained \(^{65}\text{Zn}\) with the highest amount in the cytosol followed by the microsomal fraction. The cytosol showed a gradual clearance of \(^{65}\text{Zn}\) after the injection, however, the microsomal fraction showed an increase in the \(^{65}\text{Zn}\) content around 2 h followed by a decrease.

The liver cytosol obtained from \(^{65}\text{Zn}\) dosed rats was chromatographed on Sephadex gel and it was found that approx. 90% of \(^{65}\text{Zn}\) was associated with 280 nm u.v. absorbing material of mol. wt. 10,000 to 60,000.

These studies clearly indicated the ubiquitous
distribution of i.v. injected $^{65}$Zn in the soft tissues, in different types of cells and subcellular organelles of the liver.
CHAPTER 5

DISCUSSION

Simple experiments were conducted in rats using $^{65}\text{Zn}^{2+}$ containing a known amount of carrier zinc, and the behaviour of the zinc dose was monitored by assaying the $^{65}\text{Zn}$ radioactivity. In the absence of evidence to the contrary, it was assumed that the radioactive atoms of zinc were not discriminated by the body mechanisms from the natural non-radioactive dietary zinc isotopes ($^{64}\text{Zn}, ^{66}\text{Zn}, ^{68}\text{Zn}$). It was also assumed that the conditions under which the animal experiments were conducted would not alter the zinc metabolism in the test animals to a degree greatly different from that of normals. However the test conditions have been clearly defined in order that the experiments could be repeated and the interpretations re-evaluated when the body mechanisms are better understood.

The test dose of zinc (0.2 μg) used in these experiments in rats represented a small quantity of zinc in comparison with the normal, daily dietary intake (approx. 400 μg). Assuming the blood volume of test rats to be approx. 7% of body weight, the normal zinc content in the blood 4 μg zinc/ml, and the plasma zinc to be 12% of blood zinc, it can be calculated that the total dose given intravenously was approx. 2% of the plasma zinc and approx. 0.3% of the total blood zinc. Therefore the total dose represents a very small increase in the plasma zinc level, and it can be referred to as a sub-physiological dose. The intravenous administration of $^{65}\text{Zn}$ in sub-physiological amounts can be expected to result in its incorporation into the normal
metabolic pattern of endogenous zinc. Therefore the following discussion refers to normal physiological and biochemical aspects of zinc metabolism in the rat.

The whole body counting showed that the zinc in the body is removed by at least two rate processes described by two exponential components ($t_{0.5} = 2.5$ days and $t_{0.5} = 73$ days). Of these the rapid component operates immediately after zinc dosing. In the study of Ballou and Thompson (1961) using high doses of zinc they obtained a substantially larger value for this initial process ($t_{0.5} = 6$ days) and when considered with the present study this may indicate that the body has a limited capacity to excrete zinc.

The elimination of zinc from the body is brought about by excretion in faeces and urine, and this has been shown by various workers using zinc assays on faeces and urine of normal animals, and on test animals injected with $^{65}$Zn containing high amounts of carrier zinc. The present experimental results with trace amounts of zinc are in good agreement with these findings. The major route of zinc excretion is the faeces, although $^{65}$Zn was detectable in urine at all times at approx. one hundredth of the amount detected in faeces. Although only small amounts of $^{65}$Zn were excreted in the urine in this study, a comparison with the findings of McIsaac (1955) shows that the urinary excretion of $^{65}$Zn is significantly higher after the intravenous administration of comparatively high doses of zinc. Mechanisms that regulate zinc excretion may operate in the gastro-intestinal tract and the kidney to control the quantities of zinc excreted.
under normal conditions. Increased urinary excretion of zinc may occur when systemic zinc levels are high, as after intravenous administration, and the capacity of the gastro-intestinal tract for the elimination of zinc is saturated.

In the present investigations, the amount of $^{65}\text{Zn}$ recovered in faeces and urine was greater than the whole body loss as calculated from whole body counting studies. The lack of correlation between the body content of $^{65}\text{Zn}$ and the excretion data is attributed to the errors inherent in the counting of the whole body radioactivity due to changes in geometry associated with tissue redistribution of $^{65}\text{Zn}$. When whole body clearance of $^{65}\text{Zn}$ was estimated using faecal $^{65}\text{Zn}$ excretion, the $t_{0.5}$ values obtained were considerably lower than those obtained by whole body counting. It is considered that the values obtained from the excretion data more accurately represent the $t_{0.5}$ of the rate processes involved ($t_{0.5} = 1.3$ days and $t_{0.5} = 14$ days).

Faecal excretion of $^{65}\text{Zn}$ was significantly retarded in test animals treated orally with non-toxic doses of non-radioactive zinc as ZnSO$_4$. Evidence is available from the studies of Miller et al. (1970) in cattle, that increased dietary zinc brings about a decreased absorption of an oral tracer dose of $^{65}\text{Zn}$, yet results in increased stable zinc levels in tissues. The decreased absorption in Miller's study may have been due to dilution of $^{65}\text{Zn}$ by stable zinc, resulting in decreased absorption of $^{65}\text{Zn}$. However, the increased tissue deposition indicated that the total zinc absorption from the gut was
increased and tissue accumulation resulted. Similarly in intubation of ZnSO₄ in the present study, although the fraction of zinc absorbed may have decreased, there may have been an increase in the total amount of zinc absorbed with a concomitant rise in tissue zinc levels. This would lead to the lowering of the specific activity of tissue $^{65}$Zn, which could result in the decreased $^{65}$Zn excretion seen in this experiment, where a sub-physiological dose was used intravenously. With regard to zinc absorption and excretion, homeostatic mechanisms have been postulated by Cotzias et al. (1962). These mechanisms were observed with high levels of test doses of zinc and hence the present result obtained with a sub-physiological dose do not contradict the existence of such mechanisms.

It is also possible that the ZnSO₄ intubation may have stimulated the synthesis of a protein such as the liver zinc binding protein, described by Davies et al. (1973). They demonstrated the synthesis of this protein in rats after a single intraperitoneal injection of ZnSO₄. The daily dosing of rats with ZnSO₄ in the present studies could have maintained the concentration of this protein in high amounts sufficient for the binding of the tracer dose of $^{65}$Zn, which may have retarded its excretion.

Although analytical grade ZnSO₄·7H₂O was used in the present experiment, the ZnSO₄ had an assay value of 0.001% for Cd (quoted by the manufacturer), and therefore in this experiment the animals might have received upto 0.02μg Cd per day by intubation. It is known that trace amounts of Cd
have a retarding effect on the faecal excretion of $^{65}$Zn in mice (Cotzias et al., 1962), due to the synthesis of metallothionein (Shaikh & Lucis, 1970). The occurrence of metallothionein in mammalian liver and renal cortex under normal conditions, due to life-long accumulation of Cd in these tissues, was shown by Shaikh and Lucis (1972). This protein also contains zinc in addition to Cd and can also bind zinc (Pulido et al., 1966). Thus the presence of Cd in test ZnSO$_4$ soln. used in this study may also have resulted in the retarded $^{65}$Zn excretion due to the synthesis of metallothionein in the test animals. Rats in this experiment excreted normal quantities of $^{65}$Zn in the urine and therefore the increased non-radioactive zinc levels in the body failed to alter $^{65}$Zn levels in urine. This also may be due either to protein binding of $^{65}$Zn or to lowering of the specific activity of the $^{65}$Zn.

The investigation of $^{65}$Zn in the exocrine secretions of the pancreas and liver showed that the secretions contributed nearly 30% of the total faecal $^{65}$Zn. It is therefore possible that the other 70% of the faecal $^{65}$Zn was excreted by the gastro-intestinal tract itself. The pancreatic fluid contained more $^{65}$Zn than bile and in this respect these results compared favourably with the findings on the dog (Montgomery et al., 1943). $^{65}$Zn excretion in pancreatic fluid occurred within one hour of $^{65}$ZnCl$_2$ injection, and increased to maximum levels (in parallel with the $^{65}$Zn levels in the pancreas) and then declined rapidly.
The pattern of $^{65}\text{Zn}$ excretion in bile was more complex and also constituted an important source of faecal zinc. $^{65}\text{Zn}$ levels in bile showed fluctuations in a rhythmic manner with the maximum levels in the morning and the minimum levels in the night. This variation in $^{65}\text{Zn}$ level was not directly related to the dietary intake of stable zinc. The onset of lowering of the radioactivity in bile began before the food was offered to the animals. Therefore it is likely that the diurnal variation in $^{65}\text{Zn}$ levels in bile is a true biological phenomenon occurring in the liver. The diurnal variation in biliary $^{65}\text{Zn}$ excretion may reflect,

(a) The anabolism and catabolism of zinc containing compounds in the liver,

(b) The excretion of zinc containing compounds in bile.

The bile obtained from $^{65}\text{Zn}$ dosed rats was investigated for $^{65}\text{Zn}$ containing compounds. The techniques employed were essentially those used for the study of proteins, and the separation of the biliary components was attempted in a manner to affect minimally the chemical nature of the compounds.

The ultrafiltration experiment demonstrated the absence of free ionic $^{65}\text{Zn}$ or $^{65}\text{Zn}$ bound to small molecules which could be removed with the ultrafiltrate. It is possible that the $^{65}\text{Zn}$ could be held in mixed ligand complexes, in which the $^{65}\text{Zn}$ is bound to low mol. wt. compounds which in turn are bound to macromolecules. If these were present, the association was not disrupted by thermal agitation at temperatures of 4-29$^\circ$. Therefore the result of this experiment
clearly showed that zinc in bile does not exist as free ionic zinc normally. The findings of exhaustive dialysis of bile agreed with this result and indicated that $^{65}\text{Zn}$ in bile is in a strongly bound form which could, however, be removed by EDTA at a slow linear rate. The number of binding sites for $^{65}\text{Zn}$ cannot be estimated in this system, but the linear removal may indicate a single binding site or a number of binding sites of similar affinity.

In Sephadex chromatography, $^{65}\text{Zn}$ in bile was eluted with 280nm absorbing material. The majority (75%) of the $^{65}\text{Zn}$ was eluted from the column just prior to the total bed volume of the gel, but the $^{65}\text{Zn}$ peak appeared just before the 280nm absorbing peak (i.e. the peak positions did not coincide) suggesting that $^{65}\text{Zn}$ was bound to compounds of mol.wt. greater than 5000, present in the low mol. wt. peak. The compounds in this 280nm peak were not resolved well owing to the wider fractionation range of the Sephadex G-200 gel (5000-200,000). The majority of the bile pigments were also found in this low mol. wt. region. Here the bile pigments peak corresponded well with the 280nm peak but not the $^{65}\text{Zn}$ peak. Hence it is evident that the trace amounts of zinc in bile are not exclusively bound to pigment molecules, although, it has been observed by Barrowman et al. (1973) that $^{65}\text{Zn}$ can bind to bilirubin in bile when higher zinc doses are administered to rats. In the present study approx. 25% of the $^{65}\text{Zn}$ in the column eluates corresponded with other biliary macromolecules, some of which may have been proteins of blood serum normally present in bile. It is known that human bile
contains a number of zinc metalloenzymes such as lactate dehydrogenase and alkaline phosphatase in trace amounts. These may be present in rat bile also, and their labelling with $^{65}\text{Zn}$ would account for some of the labelled macromolecules seen in Sephadex column eluates.

The Sephadex column method was not suitable for the mol. wt. determination of the major $^{65}\text{Zn}$ binding component in bile as it was considered that the elution of the low mol. wt. 280 nm absorbing peak was retarded in this system due to column contraction. The column contraction may have been due to the aromatic substances, including pyroles, in bile which have a high affinity for the Sephadex gel. For this reason a thin layer gel filtration system was used for the mol. wt. determination. Here, owing to the high sensitivity of the detection method, small quantities of bile could be used for chromatography. According to this method the major $^{65}\text{Zn}$ binding component of bile had a mol. wt. of approx. 10,000. It is possible that some retarded migration of this fraction may have still occurred, but to a lesser degree.

The immuno technique confirmed the results of the Sephadex experiments showing that the major $^{65}\text{Zn}$ binding component in bile is a protein. It does not contain a serum analogue with similar antigenic properties, showing that the low mol. wt. zinc binding protein is present only in bile.

In the paper electrophoretic study, where the biliary components were subjected to a smaller degree of separation, a number of other compounds such as amino acids, glucuronic acid, bilirubin and cholesterol were also found to be associated with or had the same electrophoretic mobility as this
protein. If these compounds are truly associated with this protein then it may serve as a carrier protein for other biliary metabolites as well as zinc. A number of preliminary experiments using ethanol at 4° to precipitate biliary protein brought about the dissociation of some of the $^{65}$Zn. A possible explanation of this is that $^{65}$Zn in bile is held in a mixed ligand protein complex, rather than bound to protein alone, and the precipitation of the protein moiety led to the dissociation of this complex.

The presence of a low mol. wt. $^{65}$Zn binding protein in rat bile has been shown recently by Barrowman et al. (1973), but the presence of a macromolecular complex in rat bile does not appear to be reported. However, in human hepatic and gall-bladder bile Bouchier and Cooperband (1967) have shown the presence of a macromolecular complex of mol. wt. 11,000 to 20,000 which is composed of cholesterol (14%), phospholipids (16%), bile salts (66%), bile pigments (3%) and protein (0.5%). The functions of this protein may be several, involving solubilisation, transport and detoxication of hepatic metabolites. The present studies have shown that this protein also binds zinc, and therefore it may serve to transport zinc in bile. The complexing of zinc with this protein may be fortuitous but on the other hand, it may be a function of the liver cell to incorporate zinc into a protein complex for excretion. It appears that the incorporation of zinc into this macromolecular complex facilitates zinc excretion from the liver cell to the external environment. It has not yet been
established whether the level of zinc-biliary-protein in bile varies diurnally, although some components of this protein complex such as cholesterol and lipids are known to vary diurnally. However, since all the $^{65}$Zn in bile has been shown to be protein bound, it is likely that the level of the biliary protein itself also varies diurnally.

Just as the physiology of the pancreas and the liver differ with regards to zinc excretion, so the chemical form of zinc in their secretions differ, also. When pancreatic fluid obtained from $^{65}$Zn dosed rats was subjected to gel chromatography similar to bile, $^{65}$Zn was eluted in association with proteins of mol. wt. 33,000 - 36,000. It is known that the pancreas elaborates carboxypeptidase (mol. wt. 34,000) and the $^{65}$Zn was probably eluted with this protein. Therefore $^{65}$Zn in the blood is taken up by the exocrine pancreas, rapidly incorporated into carboxypeptidase and secreted in the exocrine secretions.

The turnover of $^{65}$Zn in a number of soft tissues was investigated on a time-course basis, after the intravenous injection of $^{65}$ZnCl$_2$. Three aspects of the tissue distribution can be considered:

(a) $^{65}$Zn concentration in tissues,
(b) time taken to reach the maximum $^{65}$Zn levels,
(c) $^{65}$Zn clearance.

The pancreas, liver, kidney and duodenum constituted one group (group A) and contained the highest $^{65}$Zn concentration.
over 24 h. The spleen, stomach, lungs and cardiac muscle formed another group (group B) and these contained intermediate $^{65}$Zn levels over 24 h. Blood cells, voluntary muscle, testes and the prostate gland formed the third group (group C) and these contained the lowest $^{65}$Zn levels over 24 h. This tissue classification, based on the result of the sub-physiological zinc dose, compares well with the tissue classification (see Introduction) formulated on the basis of the results of other workers using $^{65}$Zn containing high amounts of stable carrier zinc. However the present result does not correlate with the tissue content of endogenous zinc, possibly because of tissue differences in the rate of turnover of zinc.

Significant differences of tissue $^{65}$Zn levels were shown by individual animals from 10 min to 6 h. These differences were most pronounced in the pancreas, liver and kidney, the organs with the highest $^{65}$Zn concentrations. These differences may be attributed to anaesthesia-induced blood flow variations. The double exposure to ether vapour of the animals killed within a short time after injection of $^{65}$Zn may have caused profound variations in the amount of $^{65}$Zn transported to these tissues. Also the injection of $^{65}$ZnCl$_2$ may have been traumatic, and the rats may have reacted differently to the 0.1M-HCl in the injection. In the absence of individual variations in the animals killed later (i.e. 1-10 days) it is reasonable to attribute the initial differences to the factors mentioned above.

Group A tissues each had a characteristic $^{65}$Zn concentration curve. Although the peak concentrations
of $^{65}$Zn in pancreas, liver and kidneys were reached at different times after injection, the $^{65}$Zn was rapidly cleared from these tissues, and this may well be related to their function regarding zinc metabolism. The pancreas showed a maximum $^{65}$Zn level around 2 h and the maximum $^{65}$Zn secretion in pancreatic juice corresponded with this peak. The total amount of $^{65}$Zn in the whole organ at 2 h (approx. 3% of dose) decreased to 1% at 24 h. An amount of $^{65}$Zn (3-4% of dose) greater than the decrease in tissue content, was recovered in the pancreatic fluid, within 24 h in cannulated rats injected with a similar dose of $^{65}$Zn. Therefore the pancreas extracted $^{65}$Zn from the blood for secretion into the duodenum.

In the liver the lowering of $^{65}$Zn content from the peak value at 20 min (approx. 40% of dose) to the value at 24 h (15% of dose) was not entirely due to $^{65}$Zn secretion in bile over this period, which was about 1-2% of the dose. However, the lowering of hepatic $^{65}$Zn content from 24-48 h could be attributed to biliary $^{65}$Zn secretion alone since the decrease in liver content of $^{65}$Zn corresponded to the amount in bile. Therefore the majority of $^{65}$Zn present in the liver initially, returns to the blood stream within a short time after injection and this may be responsible for the slight increase in the plasma $^{65}$Zn level observed at 2 h. Also at 2 h a slight increase of $^{65}$Zn levels in the duodenum, pancreas and stomach were observed and this may in turn be due to the increase in plasma $^{65}$Zn. Thus the major fraction of $^{65}$Zn in the liver may finally be excreted by these tissues,
as it was found that more than 80% of the total dose administered was excreted in the faeces in 25 days. It is therefore likely that the liver serves an important function in zinc distribution, probably by two independent pathways, one of which results in biliary excretion of zinc, and the other which results in its elimination from the liver into the blood stream.

In the kidneys the peak level of $^{65}$Zn was reached soon after injection of $^{65}$ZnCl$_2$ and was probably related to the blood flow to this organ. The kidneys contained approx. 7% of dose at 6 h, and decreased to 5.5% at 24 h. However, only a small quantity (less than 0.5% of dose) of $^{65}$Zn was recovered in the urine. As with the liver, the kidneys appear to release the major proportion of their $^{65}$Zn into the blood stream and only a small percentage is excreted in the urine.

The initial uptake and clearance of $^{65}$Zn from the duodenum was different from the pancreas, liver and kidneys. The fluctuations in $^{65}$Zn levels observed in the duodenum may be due to the elimination of $^{65}$Zn in the duodenal secretions and reabsorption of $^{65}$Zn from the duodenal lumen.

The testes, prostate gland, voluntary muscle and the formed elements of blood contained the lowest $^{65}$Zn levels of the tissues studied. Testes, blood cells and muscle contained a low $^{65}$Zn level initially, and accumulated $^{65}$Zn over a considerable period of time. Lactate dehydrogenase and malate dehydrogenase are zinc metalloenzymes present in skeletal muscle but it is not clear whether $^{65}$Zn accumulation
in muscle is entirely due to the incorporation of $^{65}\text{Zn}$ into these enzymes. In the testes endogenous zinc content has been found to increase during the maturation of the testes (Parizek et al., 1966) and also zinc is present in high amounts in spermatozoa and seminal plasma. $^{65}\text{Zn}$ accumulation in the testes in the present study is consistent with this data. The initial levels of $^{65}\text{Zn}$ in the formed elements of blood may be due to an initial diffusion of $^{65}\text{Zn}$ into the erythrocytes but the later slow rise may be due to $^{65}\text{Zn}$ incorporation into newly formed erythrocytes and leucocytes. Erythrocytes have been shown to contain the zinc metalloenzyme carbonic anhydrase and the leucocytes zinc-containing alkaline phosphatase, and $^{65}\text{Zn}$ accumulation in the formed elements may be related to the incorporation of $^{65}\text{Zn}$ into these enzymes.

The ZnSO$_4$ dosing by gastric intubation significantly increased the 24 h $^{65}\text{Zn}$ concentration (due to i.v. injection) in the liver, kidney, pancreas and duodenum. This result is in agreement with the findings of Miller et al. (1970), who found that feeding of added dietary non-radioactive zinc as ZnSO$_4$ (600 p.p.m.) caused an increase in the $^{65}\text{Zn}$ concentration in similar tissues in cattle. The increased $^{65}\text{Zn}$ levels found in tissues in the present study may be due to the retarded faecal excretion of $^{65}\text{Zn}$. The suggestions made to explain the decreased $^{65}\text{Zn}$ excretion in ZnSO$_4$ treated rats (see pp. 155-157) are also consistent with this finding. Tissues with a slow zinc turnover, such as the spleen and prostate gland, contained significantly
lower $^{65}\text{Zn}$ concentrations in contrast to the tissues with a higher zinc turnover. This may be due to:

(a) a decrease in the available $^{65}\text{Zn}$ due to an increased affinity for $^{65}\text{Zn}$ in other tissues such as the liver, kidney, pancreas and duodenum,

(b) an absence in the spleen and prostate gland of the synthesis of the postulated zinc binding protein,

(c) a higher level of accumulated stable zinc in the prostate gland, caused by long term intubation of ZnSO$_4$ which may have prevented the normal uptake of $^{65}\text{Zn}$.

The ZnSO$_4$ intubation did not affect the 24 h $^{65}\text{Zn}$ levels in plasma significantly, suggesting that the plasma turnover of zinc is not affected by increased ingestion of zinc.

The cellular localisation of $^{65}\text{Zn}$ in the pancreas, duodenum, liver and prostate gland was studied in an attempt to obtain further information on the behaviour of zinc in these tissues. The tail portion of the pancreas, which usually contains more islets of Langerhans, was examined to see whether there was a differential concentration of $^{65}\text{Zn}$ in comparison with the exocrine tissue. However, both these tissues contained a similar concentration of $^{65}\text{Zn}$. This finding with the sub-physiological dose of zinc was different from the result obtained in the rat by McIsaac (1955), using
a higher dose of zinc (500 μg). McIsaac found more $^{65}$Zn in the exocrine tissue initially, which later decreased below that of the endocrine tissue, and this result agrees with the known $^{65}$Zn loss in the exocrine secretions. The present study, however, shows that the normal turnover of zinc in exocrine and endocrine tissue is similar. It was also noted that within the islet of Langerhans no preferential localisation of $^{65}$Zn in either α or β cells is found. The initial $^{65}$Zn localisation observed in the basal region of the acinar cells and the later $^{65}$Zn localisation in the apical region may indicate the transport of $^{65}$Zn from the periphery of the acinus towards the central ductule that drains the acinus. This may represent the passage of $^{65}$Zn through the acinar cell prior to secretion in the pancreatic fluid. The cytoplasm at the basal region of the acinar cell is basophilic, owing to the presence of cytoplasmic RNA, involved in protein synthesis. Pancreatic carboxypeptidase may be synthesised in this region of the cell, and the localisation of $^{65}$Zn may therefore be related to its incorporation in carboxypeptidase. Synthesised proteins accumulate in the zymogen granules which are present in abundance in the apical region of the acinar cell and therefore the presence of $^{65}$Zn in this apical region may be related to the accumulation of $^{65}$Zn-carboxypeptidase.

It is known that the duodenum is an important site for the excretion of zinc and $^{65}$Zn has been recovered in the duodenal juice of dogs after intravenous administration
of $^{65}$ZnCl$_2$ (Montgomery et al., 1943), but $^{65}$Zn localisation at the cellular level in the duodenum has not been reported previously. The present study revealed that the Paneth cells of the crypts of Lieberkühn and goblet cells of villi contained more $^{65}$Zn than surrounding tissue. Therefore it is suggested that the zinc is excreted into the duodenum at two different sites, namely the crypt of Lieberkühn and villi, and the high $^{65}$Zn content in the Paneth and goblet cells is related to the zinc excretion from these cells. The maximum zinc concentration in these cells occurred at different times after $^{65}$Zn injection, the Paneth cells showing an initial high concentration and the goblet cells a later high concentration. The appearance of $^{65}$Zn in the duodenal lumen in close proximity to both Paneth and goblet cells suggests that these cells are responsible for the excretion of $^{65}$Zn. It was also noted that the epithelial cells contained trace amounts of $^{65}$Zn. It is not clear whether this $^{65}$Zn originated from the blood or whether it was due to absorption of $^{65}$Zn from the duodenal lumen. However the epithelium of the small intestine is shed frequently, and in rats it is renewed every two to three days, hence there may be a very slow loss of $^{65}$Zn in this way.

Paneth cells are known to contain high amounts of endogenous zinc (Ham, 1969), and the present study revealed that they are also capable of a high turnover of zinc. Paneth cells are known to have features of enzyme secreting cells (Bloom & Fawcett, 1962), but their precise function, and the composition of their secretion does not appear to be
known. The goblet cells unlike the Paneth cells did not contain $^{65}$Zn initially but at later times these as well as their secretions contained $^{65}$Zn. Goblet cells elaborate glycoproteins, but it not known whether these contain zinc. Zinc is required for protein synthesis, so it is possible that high $^{65}$Zn in the goblet cells could be related to glycoprotein synthesis.

The $^{65}$Zn concentration at localised spots in the centre of the lamina propria of villi appeared to be due to the presence of $^{65}$Zn in the lacteal, rather than in the blood capillaries. This may be due to the reabsorption of $^{65}$Zn from the duodenal lumen in association with lipid substances.

In the rat prostate gland the dorso-lateral portion is known to concentrate more $^{65}$Zn than the ventral lobes. Therefore the distribution of $^{65}$Zn in the dorso-lateral portion was studied by autoradiography. $^{65}$Zn autoradiographs resembled those of the thyroid gland, obtained with $^{131}$I, in that there was a great variability in the amount of radioactivity in different secretory units with some showing heavy localisation of $^{65}$Zn within the epithelium lining the glandular cavities and their exudates. The presence of high amounts of $^{65}$Zn in some follicles and the virtual absence of $^{65}$Zn in other follicles may be related to the functional state of the different follicles or due to the storage of $^{65}$Zn labelled secretions in the follicular lumen. The nature of zinc in prostatic secretions
is not known. Although the epithelial cells are known to be rich in acid phosphatase, zinc has not been shown to be related to its structure or function, and in an in vitro system it has been shown that addition of zinc to leucocyte acid phosphatase decreases the activity of the enzyme (Dechatlet et al., 1971).

The centrifugational analysis showed that all subcellular fractions of rat liver contained $^{65}\text{Zn}$ in different amounts with higher quantities in the cytosol and the microsomal fraction. However, stable endogenous zinc has been found in higher amounts in the cytosol and nuclei (Vallee, 1962). As the subcellular fractions were prepared at various times after $^{65}\text{Zn}$ injection, the relative clearance rates in the various subcellular fractions were revealed. In the nuclear and mitochondrial fractions, fairly constant $^{65}\text{Zn}$ levels (% of total homogenate) were maintained suggesting a turnover of zinc in these organelles the same as observed for the whole liver. The cytosol percentage content of $^{65}\text{Zn}$ decreased slightly over the 48 h period, whereas the microsomal fraction showed a peak at 2 h which then decreased more rapidly, than the cytosol content. This suggests that the $^{65}\text{Zn}$ was retained to some extent by the microsomal fraction or at least was not cleared at the same rate as the $^{65}\text{Zn}$ loss from the whole liver.

The cellular macromolecules that are likely to form $^{65}\text{Zn}$ protein complexes and metalloproteins were cited in Chapter 1. Although these may account in part for the presence
of $^{65}\text{Zn}$ in the subcellular fractions, the present knowledge of zinc interaction at the cellular level is undoubtedly too incomplete to offer a satisfactory explanation. The ubiquitous distribution of $^{65}\text{Zn}$ within the liver cell may be due to its interaction with the membranes of cell organelles as well as its association with several soluble proteins in the cell sap.

The turnover of $^{65}\text{Zn}$ in the microsomal fraction and cytosol can be interpreted as being at least partly due to the synthesis of zinc binding proteins, such as the known metalloenzymes and the zinc biliary protein complex, thus resulting in the intracellular redistribution and excretion of $^{65}\text{Zn}$.

In Sephadex chromatography of rat liver cytosol, $^{65}\text{Zn}$ was found to associate with a variety of cytoplasmic proteins of different mol. wt. In recent years two metalloproteins of zinc have been isolated from the liver cell cytoplasm. These are hepatocuprein isolated from human liver (Carrico & Deutsch, 1970) and metallothionein isolated from the liver cell sap of a number of experimental animals and man (Shaikh & Lucis, 1970) and these have mol. wts. of 33,000 and 10,500 respectively. Similar proteins and other proteins may account in part for the $^{65}\text{Zn}$ binding seen in the present experiments.

Pancreas and prostate gland scanning:

The experiments discussed in this thesis give some information on the behaviour of a sub-physiological tracer dose
of zinc such as that used in scanning and the results of these experiments can be used to a certain extent to assess whether radionuclides of zinc (as ZnCl₂) are suitable for scanning of the pancreas and prostate gland. The results of the present experiments may also be of use in the interpretation of the results obtained with the use of radionuclides of zinc in scanning. For the clear delineation of the pancreas and the prostate gland the most important requirement is that the radioactivity in these tissues, due to a radionuclide of zinc must be sufficiently high so as to fulfil the requirement of radioactivity detection with a minimum contribution from the surrounding tissues. The present investigations clearly indicated that apart from the pancreas, a number of tissues such as the liver, duodenum and kidneys are important for zinc metabolism, taking an active part in ⁶⁵Zn concentration, incorporation into macromolecules and excretion. The size of the liver, and the anatomical position of the pancreas in relation to the liver and duodenum become most critical as these would show high amounts of radioactivity. In order that the pancreas is better visualised by scanning, it must retain its radioactivity while the neighbouring tissues must show a clearance. However, the excretion of zinc is pronounced in the pancreas and less in the liver. The initial zinc secretion from the pancreas prevents the maintenance of zinc concentration within the gland. The radioactivity in the duodenum due to its own secretions and pancreatic fluid would present difficulties.
in delineating the head of the pancreas and tumours present in this part of the pancreas. It was believed that radionuclides of zinc would be of use in scanning for tumours of the pancreas such as the insulin secreting tumours of the islets, which are difficult to locate even at the operation (Fonkalsurd et al., 1964). But in the autoradiographic study it was seen that there was no preferential localisation of zinc in the islets of Langerhans in comparison with the exocrine pancreas or in the \( \beta \) cells in comparison with the \( \alpha \) cells and therefore it seems unlikely that the insulin secreting tumours can be visualised with the use of radioactive zinc.

The initial zinc level in the prostate gland was low, and the ratio of zinc between the prostatic tissue and blood was low, from 0-6 h after injection. Therefore this may hinder the external visualisation of the gland, initially. Although there is a long term accumulation of zinc in the prostate gland, owing to the accumulation of zinc in muscle and the excretion of zinc in the faeces, the radioactivity in the neighbourhood of the prostate gland rises. This again may present problems in the scanning of the gland at later times after injection of radionuclides of zinc. The autoradiographic investigations on the rat and human (human results not reported in the thesis) prostate gland showed a non-uniform zinc distribution in different areas of the gland, probably related to the functional state. Zinc was located in this study within the lumen of secretory units perhaps stored with the secretions, rather than over

-174-
cellular areas. Owing to the lack of a uniform distribution it may be difficult to outline the normal gland. In the human, the carcinomatous prostate gland has been shown to have a low uptake in the affected areas, and in view of the absence of a relatively high concentration in all normal tissue, these tumours may not be visualised with radioactive zinc.

Although the above discussion refers to the behaviour of ionic zinc (Zn$^{2+}$), its metabolism may differ when the chemical nature of zinc is altered and such radio-Zn-preparations may be worth testing.

**Oral ZnSO$_4$ therapy:**

An experiment was conducted to study the excretion and tissue distribution of a sub-physiological tracer dose of intravenously administered zinc in rats given an equivalent of a therapeutic dose of ZnSO$_4$·7H$_2$O (given orally in humans) for 30 days. During this period of ZnSO$_4$ intubation the test rats did not show any symptoms of zinc toxicity. However the endogenous test zinc was excreted more slowly and the zinc dose was retained longer in the major organs of zinc metabolism. While it is clear that the continued ZnSO$_4$ treatment caused a slower turnover of zinc in the body, the reason for the increased tissue retention of zinc was not investigated. But in view of the recent findings of ZnSO$_4$-induction of zinc binding protein synthesis it was suggested that the decreased turnover could be due to the synthesis of zinc binding proteins in the liver, pancreas, duodenum, and kidneys.
CHAPTER 6

TISSUE DISTRIBUTION AND EXCRETION OF GALLIUM

6-1 Introduction

In recent years compounds of gallium have been used in diagnostic medicine in the United Kingdom. Gallium has been studied with regard to its behaviour in the vertebrate body including its toxicology and this has been reviewed in Chapter 1. The availability of this element in carrier-free form as $^{67}\text{Ga}$ has permitted the study of its tissue distribution and blood transport reported in this Chapter.

$^{67}\text{Ga}$ is a moderately short-lived radionuclide of $t_{0.5}=3.24$ days, and it decays to stable $^{67}\text{Zn}$ by electron capture. The electron capture process results in the release of the following gamma-ray quanta:

<table>
<thead>
<tr>
<th>Energy (MeV)</th>
<th>Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.093</td>
<td>40</td>
</tr>
<tr>
<td>0.184</td>
<td>24</td>
</tr>
<tr>
<td>0.296</td>
<td>22</td>
</tr>
<tr>
<td>0.388</td>
<td>7</td>
</tr>
</tbody>
</table>

By Lederer et al. (1967)

The gamma-rays, particularly, the 0.184 and 0.296 MeV with a total percentage abundance of 46% are suitable
for the assay of $^{67}$Ga in biological samples, by scintillation counting. The Auger electrons, 0.1 to 9.5 keV have been shown satisfactory for micro-assay of $^{67}$Ga at subcellular level by autoradiography of thin histological sections. (Swartzendruber et al., 1971).

6-2 Experimental techniques

6-2-1 Animal experimentation

Normal Wistar albino rats (approx. 200g) were used for metabolic studies and as blood donors for protein binding studies. These as well as tumour bearing animals were housed and maintained during the pre-experimental and experimental periods as described in Section 2-1-1.

Wistar rats transplanted with two types of tumours, RIB5 and SSB1 were used. RIB5 tumour is a benzpyrene-induced fibrosarcoma maintained by successive subcutaneous transplantations. These grew to reach a mean diameter of 9 to 10 mm two weeks post transplantation. Over the size ranges used it had an average volume doubling time of about a day. RIB5 is an anaplastic tumour with no differentiated cells (Denekamp, 1968). The tumour cells infiltrate the underlying muscle and skin in the immediate neighbourhood and develop multifocal necrosis at a very early stage of growth.

SSB1 tumour originated as a spontaneous fibrosarcoma in a Wistar rat and has been maintained by successive transplantations in rats. It is a well differentiated tumour with a doubling time of 3.5 days. This is a relatively slow
growing tumour, and takes approx. a month to reach 9 to 10 mm post transplantation.

RIB5 tumours were transplanted subcutaneously in the flank of Wistar rats (160g) with the usual trochar technique. SSB1 tumours were grown by implanting 2 to 3 mm cubes of tumour, subcutaneously, in recipient Wistar rats (150g).

The normal and tumour bearing rats were injected in the caudal vein (see Section 2-1-4) with approx. 9μCi of $^{67}$Ga citrate.

6-2-2 Whole body counting, excretion and gross tissue distribution

A group of 15 normal rats were injected with 9μCi of $^{67}$Ga citrate per animal and the dose received by each animal was obtained by whole body counting (see Section 2-4-2). At regular 24 h intervals after dosing, the rats were sacrificed in groups of three each day for tissue distribution studies, and prior to killing the amount of $^{67}$Ga left in the body was estimated by whole body counting.

Immediately after death tissues were removed from the carcass. Tissues similar to those taken for the $^{65}$Zn study were prepared in a manner similar to the description in Section 4-2-1, and these included:

- Blood
- Lungs
- Liver
- Thigh muscle
- Duodenum
- Kidney
- Spleen
- Testes

In addition to these tissues, the whole brain and a representative sample of skin with hair and the left femur were taken.
The brain was exposed by a median scissor cut of the cranial capsule and parting the bony plates to either side. The brain was lifted off with a spatula and transferred to a counting vial. The skin with hair was taken from the back of the animal. To obtain the femur, skin and muscles were teased from the left thigh. The bone was cut in two pieces and the marrow was removed with a jet of saline.

Tissue distribution of $^{67}$Ga was also studied in a group of 15 rats bearing 9 - 10 mm SSB1 tumour. These were dosed with 9μCi of $^{67}$Ga citrate. Tissues for $^{67}$Ga assay included tissues mentioned above and the tumour.

In the case of spleen, left kidney, lungs, femur, brain and left testicle, the whole tissue was assayed.

For blood plasma, liver (tissue cube from the anterior lobe), duodenum, thigh muscle and skin, a 1 g sample was assayed. Tumour was bisected and the halves placed in two counting vials. All tissues were packed in the bottom of counting vials so that the change of tissue volumes was minimal. Radioactivity due to $^{67}$Ga was assayed in a gamma-ray spectrometer (see Section 2-2-3) and the counting rates were corrected for radioactive decay, and background counting rates.

In another experiment six rats, two normal, two RIB5 tumour bearing and two SSB1 tumour bearing rats were injected with 9μCi of $^{67}$Ga citrate. These were housed in metabolism cages and samples of faeces and urine collected daily for 5 days. $^{67}$Ga in urine and faeces was assayed by gamma-ray spectrometry.
6-2-3 67Ga distribution in transplanted tumours

The rats bearing RIB5 tumour and SSB1 tumour were intravenously injected with 10μCi of 67Ga citrate, when the tumours reached a diameter of 5 - 13 mm. The rats were killed at 2 h and 1,2,3,4 and 5 days after 67Ga injection. The tumours were quickly removed, frozen in n-hexane, and 20μ thick histological sections cut across the tumour, in a cryostat and picked up on pre-cooled (approx. 0°) microscope cover glasses. The tissue containing cover glass was secured on to a 5μ thick Melinex sheet (ICI Ltd.), and Kodirex films were exposed to the tissue at 4° for 4 days. Fogging of the film due to biological material was prevented by the interlayer of Melinex between tissue and film. The film was processed and the photographic image analysed in relation to the histological section.

6-2-4 67Ga localisation in tumour cell

A group of 5 rats bearing RIB5 tumours, approx. 10 mm diameter were injected with 10μCi of 67Ga citrate in the caudal vein. Twenty four hours later the rats were killed and the tumour removed. Approx. 5μ thick sections were autoradiographed according to the method described in Section 4-2-2. The length of exposure was 5 to 7 days.

6-2-5 Investigation of 67Ga binding serum proteins

67Ga protein binding was studied with blood samples obtained from normal rats and patients with confirmed
malignant disease. Both species received a similar dose equivalent (0.045 μCi/g body weight) of carrier-free 67Ga citrate. The patients were injected with approx. 2mCi of 67Ga citrate in the antecubital vein. The total volume of the injectate was about 2 ml and was forced into the vein as a bolus. The rats were injected in the tail vein with 9μCi of 67Ga citrate. Blood samples were obtained 3, 24 and 72 h after injection, from rats by cardiac puncture and in humans from the antecubital vein in the other arm. Blood samples were allowed to clot in sterile tubes at 37º and the resulting sera were taken for the following investigations:

Ultrafiltration (see Section 3-2-2)
Exhaustive dialysis (see Section 3-2-3)
Zone electrophoresis
Electro-immunodiffusion

The zone electrophoresis technique used was as follows:

Electrophoresis was conducted in a horizontal apparatus using Cellulose Acetate Membranes (CAM) as a supporting medium. Three buffer solns. were used in separate experiments and the operating conditions for the respective buffers are shown in the Table 6-5. Approx. 10 μl of serum was applied over the CAM strip soaked in the appropriate buffer soln., and subjected to electrophoresis for 2 h. 67Ga citrate (10 μl) with a counting rate similar to sera was also subjected to electrophoresis as a control in a parallel strip. At the end of the run the strip was dried in a horizontal position in air and cut into 0.5 cm pieces across the length of the strip for 67Ga assay. The parallel strip was stained with Ponceau S for proteins.
The stained strips were either scanned in a chromoscanner (Joyce Lobel, London) or visually checked for protein zones. Sera obtained from normal rats and cancer patients at 3 h and 24 h after injection of $^{67}\text{Ga}$ citrate were investigated by this method.

Blood sera obtained 3 h after $^{67}\text{gallium}$ citrate injection in cancer patients were also investigated by the method of electro-immunodiffusion. This method was described previously in Section 3-2-7, and the conditions were similar except that the gel was prepared in 0.04M-tris-barbitone sodium barbitone buffer, and the electrode buffer was the same buffer soln. at half strength. Ten µl of serum was placed in the sample well, within a minimum time after collection and the proteins were separated in the first dimension by electrophoresis at 24V/cm for 45 min. The separated proteins were forced into the second dimension gel bed impregnated with a multivalent antiserum for normal human serum proteins at 8V/cm for 8 h. The electrophoresis was conducted at 4°. At the end of the run excess antiserum was washed out with 0.9% NaCl soln. and $^{67}\text{Ga}$ located by autoradiography using the AR.50 autoradiographic film (Kodak Ltd.).
Table 6-5  Electrophoretic buffers for (CAM) zone electrophoresis and operating conditions

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Constituents</th>
<th>Concentration (molarity)</th>
<th>Current (V/cm)</th>
<th>Current (mA/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phosphate</td>
<td>7.8</td>
<td>KH$_2$PO$_4$</td>
<td>$5 \times 10^{-4}$</td>
<td>25</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na$_2$HPO$_4$</td>
<td>$6 \times 10^{-3}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Citrate-phosphate</td>
<td>7.8</td>
<td>Citric acid</td>
<td>$5 \times 10^{-4}$</td>
<td>20</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na$_2$HPO$_4$</td>
<td>$9.5 \times 10^{-3}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Tris-barbitone</td>
<td></td>
<td>Sodium barbitone*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trishydroxy methyl aminomethane</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diethyl barbituric acid,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>and Sodium barbitone</td>
<td>$4 \times 10^{-2}$</td>
<td>20</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* obtained from Gelman Instrument Co., Ann Arbor, Michigan.
6-3 Materials

$^{67}\text{Ga}$ was produced in the MRC cyclotron by the nuclear reaction:

$$^{65}\text{Cu} + ^4\text{He} \rightarrow ^{67}\text{Ga} + 2\text{n}$$

(Sylvester & Thakur, 1970)

The radioactive surface of the irradiated Cu target was etched with HNO$_3$ and the resulting soln. was boiled down with conc. HCl. The residue was taken up in HCl and metal contaminants were removed by cation exchange chromatography. $^{67}\text{Ga}$ free from the major chemical impurity Cu and the radioactive impurity, $^{65}\text{Zn}$, was obtained in 10 ml of 3.5M-HCl. This soln. was evaporated to dryness and $^{67}\text{Ga}$ was taken up in a suitable volume of 3.8% sodium citrate soln. The doses were prepared from this stock soln. by dilution with 3.8% sodium citrate soln. so that 9μCi was contained in 0.2 ml for injection into rats and 2.0mCi in 2.0 ml for injection into patients. The amount of gallium in the respective injections was $2 \times 10^{-12}$ and $4 \times 10^{-10}$ μmol.

Rabbit antisera for human serum proteins were obtained from Hoechst Pharmaceuticals Ltd., Brentford, Middx. and Cellulose Acetate Membranes for zone electrophoresis was purchased from Hawksley & Son Ltd., Lancing, Sussex.
A group of 15 normal rats and 15 tumour bearing rats were injected with 9µCi of $^{67}$Ga citrate and the radioactivity was counted in the whole body counter. The amount of $^{67}$Ga injected was assumed to be equal to 100% and the $^{67}$Ga remaining in the body was expressed as a percentage of the initial counting rate. In this experiment both tumour bearing and normal rats retained similar amounts of $^{67}$Ga, hence the results are considered together and are shown in Table 6-1.

Table 6-1 $^{67}$Ga retention in the rat

<table>
<thead>
<tr>
<th>Time after injection (days)</th>
<th>Amount of $^{67}$Ga retained (% of initial dose)</th>
<th>Mean ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>85.62</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>80.62</td>
<td>4.1</td>
</tr>
<tr>
<td>3</td>
<td>76.87</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>77.74</td>
<td>3.9</td>
</tr>
<tr>
<td>5</td>
<td>69.79</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Mean ± s.d. for 6 rats.

These results show that the tracer dose was rather slowly removed from the body.
6-4-2 $^{67}$Ga excretion in faeces and urine

Six rats injected (i.v.) with approx. 9μCi of $^{67}$Ga citrate were housed in metabolism cages. Urine and faeces samples were collected and assayed for $^{67}$Ga. The two normal rats and the four tumour bearing rats excreted similar amounts of $^{67}$Ga and the results were considered together (Table 6-2). During the first two days more $^{67}$Ga was excreted in urine than in the faeces but after two days only small amounts were excreted in urine and more $^{67}$Ga was recovered in the faeces. The amount of $^{67}$Ga recovered in faeces and urine accounted for all the loss of $^{67}$Ga seen in whole body counting.

Table 6-2 $^{67}$Ga excretion in faeces and urine (cumulative)

<table>
<thead>
<tr>
<th>Time after injection (days)</th>
<th>$^{67}$Ga radioactivity (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine + s.d.</td>
</tr>
<tr>
<td>1</td>
<td>Mean ± 9.57 ± 2.4</td>
</tr>
<tr>
<td>2</td>
<td>12.84 ± 3.3</td>
</tr>
<tr>
<td>3</td>
<td>14.05 ± 3.6</td>
</tr>
<tr>
<td>4</td>
<td>14.56 ± 3.7</td>
</tr>
<tr>
<td>5</td>
<td>15.03 ± 3.6</td>
</tr>
</tbody>
</table>

Mean ± s.d. for 6 rats.

6-4-3 Tissue distribution of $^{67}$Ga

The rats in Section 6-4-1 were killed daily, 3 from the normal group and 3 from the tumour bearing group.
A number of tissues were removed from the carcass immediately after death and the $^{67}\text{Ga}$ assayed. The results are given in Table 6-3. The tissues of tumour bearing rats contained similar amounts of $^{67}\text{Ga}$ to that contained in the tissues of normal rats, hence the results of the two groups are considered together. $^{67}\text{Ga}$ was cleared extremely slowly from blood plasma during 2 - 5 days and the concentration was almost identical to that of whole blood. When blood cells recovered in the preparation of plasma were washed free of plasma proteins and counted for $^{67}\text{Ga}$, these contained similar amounts of $^{67}\text{Ga}$ to that in the plasma from which they were separated. The highest concentration of gallium was found in the liver, spleen and kidney at 24 h but the $^{67}\text{Ga}$ in the kidney cleared rapidly. The femur, lungs, skin and testes contained lower concentrations than the above tissues but higher concentrations than muscle, duodenum and brain. On a unit weight basis, brain contained the smallest amount of $^{67}\text{Ga}$.

The uptake and loss of $^{67}\text{Ga}$ in the various tissues showed differences. The kidney, liver and spleen showed a relatively rapid clearance (Fig.6.1), while the skeletal muscle, duodenum and lungs maintained fairly constant levels of $^{67}\text{Ga}$ during the 5 days. Of the other tissues, the testes and brain (Fig.6.2) and also the femur (Table 6-3) accumulated $^{67}\text{Ga}$.

The SSB1 tumour contained a higher concentration of $^{67}\text{Ga}$ than body tissues, and showed a tendency to accumulate initially and retain $^{67}\text{Ga}$ (table 6-4). This is also evident from the
Table 6-3  Tissue distribution of i.v. $^{67}$Ga citrate in the rat
(as a percentage of the injected dose per gram tissue wet wt.)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time after injection (days)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Whole blood</td>
<td>0.120 ± 0.016</td>
<td>0.045 ± 0.009</td>
<td>0.031 ± 0.010</td>
<td>0.030 ± 0.008</td>
<td>0.021 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.118</td>
<td>0.010</td>
<td>0.055</td>
<td>0.006</td>
<td>0.034</td>
<td>0.003</td>
</tr>
<tr>
<td>Brain</td>
<td>0.016</td>
<td>0.001</td>
<td>0.027</td>
<td>0.003</td>
<td>0.034</td>
<td>0.003</td>
</tr>
<tr>
<td>Testes</td>
<td>0.178</td>
<td>0.014</td>
<td>0.185</td>
<td>0.016</td>
<td>0.195</td>
<td>0.013</td>
</tr>
<tr>
<td>Femur</td>
<td>0.217</td>
<td>0.014</td>
<td>0.261</td>
<td>0.012</td>
<td>0.275</td>
<td>0.013</td>
</tr>
<tr>
<td>Skin</td>
<td>0.175</td>
<td>0.002</td>
<td>0.205</td>
<td>0.004</td>
<td>0.224</td>
<td>0.006</td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.064</td>
<td>0.027</td>
<td>0.134</td>
<td>0.062</td>
<td>0.129</td>
<td>0.034</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.154</td>
<td>0.003</td>
<td>0.179</td>
<td>0.006</td>
<td>0.157</td>
<td>0.004</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.260</td>
<td>0.034</td>
<td>0.225</td>
<td>0.023</td>
<td>0.231</td>
<td>0.014</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.606</td>
<td>0.107</td>
<td>0.144</td>
<td>0.077</td>
<td>0.105</td>
<td>0.054</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.517</td>
<td>0.050</td>
<td>0.315</td>
<td>0.033</td>
<td>0.416</td>
<td>0.052</td>
</tr>
<tr>
<td>Liver</td>
<td>0.657</td>
<td>0.033</td>
<td>0.688</td>
<td>0.043</td>
<td>0.353</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Mean ± s.d. for six rats
Fig. 6.1. $^{67}$Ga clearance in the liver (□) kidney (▲) and spleen (▼) after injection (i.v.) of $^{67}$Ga citrate in the rat.

A mean for six rats is given.
Fig. 6.2. $^{67}$Ga accumulation in the testes (□) and brain (□) of the rat after a single intravenous dose of $^{67}$Ga citrate.

A mean of six animals is given.
constant ratio for $^{67}$Ga between tumour and plasma from 3 to 5 days (Table 6-4).

### Table 6-4 $^{67}$Ga concentration in SSB1 tumour

<table>
<thead>
<tr>
<th>Time after injection (days)</th>
<th>$^{67}$Ga concentration (% injected dose/g)</th>
<th>Tumour/Plasma (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4937 ± 0.0437</td>
<td>3.8 - 4.4</td>
</tr>
<tr>
<td>2</td>
<td>0.6397 ± 0.0588</td>
<td>10.2 - 12.3</td>
</tr>
<tr>
<td>3</td>
<td>0.7081 ± 0.0573</td>
<td>19.8 - 24.6</td>
</tr>
<tr>
<td>4</td>
<td>0.6755 ± 0.0827</td>
<td>20.2 - 23.4</td>
</tr>
<tr>
<td>5</td>
<td>0.6426 ± 0.0808</td>
<td>20.4 - 23.3</td>
</tr>
</tbody>
</table>

Mean and s.d. for three SSB1 tumours per time interval.

$^{67}$Gallium distribution in experimental tumours by gross autoradiography

Rats bearing 5-13 mm diameter RIB5 and SSB1 tumours were injected with 10μCi of $^{67}$Ga citrate. Animals were killed at 2 h and 1 - 5 days after injection. Approx. 20μ thick histological sections were autoradiographed and the areas of $^{67}$Ga localisation were studied.

The regional $^{67}$Ga distribution in RIB5 tumours varied according to the size of the tumour. At 2 h, 5 - 7 mm tumours contained $^{67}$Ga throughout the entire cross section of the tumour; however, the blood vessels present at the periphery of the tumour contained more radioactivity than...
Fig. 6.3. $^{67}$Ga distribution in experimental rat tumours.

N= areas of necrosis
BV= blood vessels

These sketches were drawn combining the histological section and the autoradiographic image due to $^{67}$Ga. The darker shade represents areas of increased $^{67}$Ga radioactivity.

(a) Approx. 5mm diameter RIB5 tumour.

Note radioactivity in the periphery of the tumour near blood vessels and in the central zone (between necrotic and vital zones) 24 h after $^{67}$Ga citrate injection.

Fig. captions continued...
(b) **Approx. 8mm diameter RIB5 tumour.**

Note $^{67}$Ga localisation in the peripheral zone (vital) and the absence of radioactivity in the central zone (necrotic) 24 h after $^{67}$Ga citrate injection.

(c) **About 12mm diameter RIB5 tumour.**

$^{67}$Ga localised as a thin band on the periphery of the tumour, 72 h after $^{67}$Ga citrate injection. Note that the major area of the tumour is free of radioactivity.

(d) **SSB1 tumour**

approx. 9 mm tumour showing $^{67}$Ga localisation in the vital areas of the tumour. 120 h after $^{67}$Ga citrate injection.
other areas of the tumour. At 24 h these tumours consistently showed a ring of radioactivity a few mm inner to the peripheral ring of blood vessels. (Fig. 6.3.a). With larger tumours (10 - 13 mm), 2 h after injection the majority of radioactivity was again located as a narrow band in the periphery of the tumour with the central areas being completely free of $^{67}$Ga (Fig. 6.3.b). The pattern of $^{67}$Ga distribution did not differ with time in these larger tumours and at all times the majority of radioactivity was located in the periphery of the tumour. The slow growing SSB1 tumour resembled the small RIB5 tumour with regards to $^{67}$Ga distribution. Both RIB5 and SSB1 showed the absence of radioactivity in the areas of necrosis, which in the latter tumour is usually less (Fig. 6.3.c & d).

6-4-5 $^{67}$Ga uptake by the RIB5 tumour

Approx. 5μ thick histological sections of 10 - 15 mm RIB5 tumours obtained 24 h after $^{67}$Ga injection were autoradiographed, and these showed very little radioactivity within the blood vessels. More $^{67}$Ga was associated with the cellular areas of the tumour. (Fig 6.4). The Ag grain density in the necrotic areas was lower than in the vital zone of the tumour. However, groups of cells scattered in the necrotic zone often showed abundant Ag grains. In the peripheral zone more grains were associated with groups of cells with darkly staining nuclei containing granular cytoplasm (Fig. 6.5). Other cells in their neighbourhood with faintly outlined non-granular cytoplasm contained few Ag grains. In certain areas where the tumour cells infiltrated
Fig. 6.4. Autoradiogram showing $^{67}$Ga localisation in the RIB5 tumour. Note the relative abundance of $^{67}$Ga radioactivity in the areas densely populated with tumour cells (arrows). The blood vessel (BV) and the vascular endothelium (E) are less radioactive.
Magnification x 400.
Fig. 6.5. 67Ga localisation in the RIB5 tumour cell.

Note the presence of increased 67Ga radioactivity in the two optically intact cells (viable) shown by the arrows. (x 1000)
the abdominal muscle the lack of excess $^{67}\text{Ga}$ in the muscle and its presence in the tumour cells was noted (Fig. 6.6).

$^{67}\text{Ga}$ localisation was mainly cytoplasmic, but occasionally some nuclei showed heavy $^{67}\text{Ga}$ labelling. The results of this experiment indicated that $^{67}\text{Ga}$ concentrated predominantly in the cytoplasm of RIB5 tumour cell that appeared to be viable.

6-4-6 Ultrafiltration of $^{67}\text{Ga}$ in serum

Blood sera obtained 3, 24 and 72 h after $^{67}\text{Ga}$ injection in patients and rats were investigated by this method. Three ml fractions of serum samples were centrifuged at 2000g for 0.5 h at 4°. $^{67}\text{Ga}$ citrate diluted with 0.9% NaCl soln. was used as controls. In a separate experiment $^{67}\text{Ga}$ binding by pure human serum albumin (HSA) was investigated. A 4% (w/v) soln of HSA was mixed with $^{67}\text{Ga}$ citrate. The radioactivity in this soln. was 1.0μCi $^{67}\text{Ga}$/ml and the citrate concentration was 0.1mM. These were typical in vivo concentrations of $^{67}\text{Ga}$ and the citrate expected in man following $^{67}\text{Ga}$ citrate injection. The mixture of HSA and $^{67}\text{Ga}$ citrate was incubated with gentle agitation for 3 h at 37° prior to ultrafiltration. The results obtained for $^{67}\text{Ga}$ binding in sera and control solutions are shown in Table 6-5.

The controls gave a binding of 15%, and this is probably due to $^{67}\text{Ga}$ binding by the cellulose membrane, and the other figures were not corrected for this blank value. In sera the percentage binding of $^{67}\text{Ga}$ was increased from 3 - 72 h
Fig. 6.6. Autoradiogram showing low $^{67}$Ga concentration in the muscle and a higher concentration in the invading RIB5 tumour cells. (x 400). M = muscle.
after injection. The binding of $^{67}$Ga by rat and human sera were similar. The lower binding figure of HSA (35%) may indicate that non-albumin proteins in serum probably played an important role in $^{67}$Ga binding.

Table 6-5 $^{67}$Ga binding in blood serum

<table>
<thead>
<tr>
<th>Sample</th>
<th>% binding</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat serum</td>
<td>Human serum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean ± s.d.</td>
<td>Mean ± s.d.</td>
<td></td>
</tr>
<tr>
<td>serum 3 h</td>
<td>87 ± 4.7</td>
<td>84 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>serum 24 h</td>
<td>98 ± 5.3</td>
<td>97 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>serum 72 h</td>
<td>97 ± 3.2</td>
<td>99 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Human serum albumin</td>
<td></td>
<td>35±2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>$^{67}$Ga controls</td>
<td></td>
<td>15*** ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

* Sera of three rats per time interval
** Sera from ten patients
*** Mean of ten samples
+ Mean of six samples

6-4-7 Exhaustive dialysis of $^{67}$Ga in serum

Serum samples obtained from rats and cancer patients 3, 24 and 72 h after $^{67}$Ga citrate injection were subjected to exhaustive dialysis. Visking cellulose bags containing 1 ml of serum were dialysed against 2 litres of sterile 0.9% NaCl solution at pH 7.2. Dialysis was continued for 24 h at 4°C. In another study using sera from different subjects and 3 rats...
injected with $^{67}\text{Ga}$ citrate the effect of phosphate ions on the dialysability of $^{67}\text{Ga}$ was examined by the dialysis of the sera against a phosphate buffer, $6 \times 10^{-3}$ mM, in 0.9% NaCl soln. at pH 7.4. $^{67}\text{Ga}$ citrate containing saline solutions were dialysed as controls. The results of a typical dialysis experiment are shown below:

<table>
<thead>
<tr>
<th>Dialysing medium</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Saline</td>
<td>2</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>0</td>
</tr>
</tbody>
</table>

Mean value of 12 human serum samples and 6 rat serum samples in each buffer solution are presented. Dialysis removed the $^{67}\text{Ga}$ in the control solutions and no radioactivity was found in association with the cellulose membranes. All sera retained about a third of $^{67}\text{Ga}$ after 24 h of dialysis and this fraction could be removed by further dialysis for 6 h against phosphate buffer. Sera dialysed against the phosphate buffer lost the $^{67}\text{Ga}$ completely in 24 h. The dialysability of $^{67}\text{Ga}$ in rat sera was slightly less than that of human sera. In both species similar amounts of $^{67}\text{Ga}$ were removed from sera obtained at 3, 24 and 72 h after injection. It was not possible to check the accuracy of the above figures by counting the dialysate for $^{67}\text{Ga}$ owing to its dilution in a large volume of buffer.
Zone electrophoresis of $^{67}$Ga binding proteins in serum

Serum samples obtained from cancer patients and normal rats, 3 and 24 h after $^{67}$Ga citrate injection were investigated by this method and the electrophoretic mobility of $^{67}$Ga in serum was compared with that of $^{67}$Ga in $^{67}$Ga citrate.

In the phosphate buffer (pH 7.8) the majority (Approx. 65%) of $^{67}$Ga remained at the point of application when $^{67}$Ga citrate was subjected to electrophoresis, whilst a smaller fraction migrated towards the anode. $^{67}$Ga in both sera at both time intervals migrated with proteins in the albumin, $\alpha$ and $\beta$-globulin zones (Fig.6.7).

In the citrate-phosphate buffer (pH 7.8), $^{67}$Ga of the controls migrated towards the anode and the sera migrated as a wider zone from $\alpha$-globulin to beyond albumin at a rate slower than the $^{67}$Ga of the controls (Fig.6.8). In addition to the major $^{67}$Ga peak, a small peak was detected in the $\beta$-globulin zone.

In the barbitone buffer (pH 8.4), $^{67}$Ga of $^{67}$Ga citrate and $^{67}$GaCl$_3$ showed an anodic migration. $^{67}$GaCl$_3$ transformed from an electrically neutral species into an anode seeking species (Fig.6.9). $^{67}$Gallium in all sera migrated with albumin $\alpha$ and $\beta$-globulin zones when serum was subjected to electrophoresis for one hour (Fig.6.10), and when electrophoresis was continued for six hours, the majority of $^{67}$Ga migrated beyond the albumin zone leaving a small peak in the $\beta$-globulin zone (Fig.6.11).
Fig. 6.7. Electrophoretic migration of $^{67}\text{Ga}$ in phosphate buffer.

$^{67}\text{Ga}$ citrate (---)

$^{67}\text{Ga}$ in blood serum (-----)

(see text for experimental details)
Fig. 6.8. Electrophoretic mobility of $^{67}$Ga in citrate-phosphate buffer.

$^{67}$Ga citrate (---)

$^{67}$Ga in blood serum (—)

(see text for details)
Fig. 6.9. Anodic migration of $^{67}$Ga chloride in zone electrophoresis in barbitone buffer.

$^{67}$Ga chloride was subjected to electrophoresis for 0.5, 1, 1.5 h. Note the gradual transformation of the electrically neutral species to an ionic species. $^{67}$Ga of gallium citrate also behaves as an anode seeking species under similar conditions.
Fig. 6.10. A typical electrophoretic separation of $^{67}$Ga in $^{67}$Ga serum in barbitone buffer.

Ten μl of a serum sample obtained from a patient 24 h after $^{67}$Ga citrate injection was subjected to zone electrophoresis in barbitone buffer, pH 8.4, at 10V/cm and 0.5 mA/cm, for one hour. $^{67}$Ga radioactivity (---)

A parallel strip stained for proteins with Ponceau S is shown.
Fig. 6.11.  Electrophoretic dissociation of serum protein bound $^{67}$Ga due to prolonged electrophoresis.

A serum sample (10ml) obtained from a cancer patient 24 h after $^{67}$Ga citrate injection was subjected to zone electrophoresis in barbitone buffer (pH 8.4) at 20V/cm for 6 h. $^{67}$Ga radioactivity (---)
Sera dialysed for 24 h against saline (see Section 6-4-7) were subjected to electrophoresis, these contained $^{67}\text{Ga}$ in the $\beta$-globulin zone only.

These investigations revealed that the electrophoretic migration of $^{67}\text{Ga}$, when $^{67}\text{Ga}$ serum was subjected to electrophoresis was different from that of free $^{67}\text{Ga}$ citrate and the presence of serum constituents delayed $^{67}\text{Ga}$ migration in citrate-phosphate and barbitone buffers and accelerated $^{67}\text{Ga}$ migration in phosphate buffer. Sera obtained from rats and cancer patients showed a similarity in $^{67}\text{Ga}$ migration and no differences were detected between 3 and 24 h samples.

6-4-9 Identification of $^{67}\text{Ga}$ binding proteins by electroimmunodiffusion

Blood serum samples obtained from cancer patients 3 h after $^{67}\text{Ga}$ injection were investigated by this technique using rabbit antiserum to normal human serum. $^{67}\text{Ga}$ in these preparations was located by autoradiography and protein peaks made visible with Ponceau S. $^{67}\text{Ga}$ citrate controls were investigated similarly.

$^{67}\text{Ga}$ in $^{67}\text{Ga}$ citrate migrated to the very edge of the gel near the respective anodes leaving the major area of the gel free of radioactivity. Blood sera showed at least 30 distinct protein peaks. $^{67}\text{Ga}$ was detected with two protein peaks by autoradiography (see Fig. 6.12) and these proteins were identified on the basis of their position as transferrin and haptoglobin. Monospecific antisera for transferrin and haptoglobin were incorporated separately into the second
dimension gel bed and the sera were investigated by electro-immunodiffusion and autoradiography. Transferrin and haptoglobin peaks immunoprecipitated contained $^{67}$Ga and the two $^{67}$Ga labelled protein peaks immunoprecipitated with multivalent antiserum occupied similar positions on the gel plate. Serum samples obtained from 12 patients consistently showed $^{67}$Ga labelling to transferrin and haptoglobin.

Fig. 6.12. $^{67}$Ga binding proteins in human blood revealed by electro-immunodiffusion and autoradiography.

Transferrin = slim peak
Haptoglobin = flat peak
A tracer dose of $^{67}$Ga citrate was used to study the mode of transport of this element in blood of humans and the rat, and its tissue distribution and excretion in the rat. Ultrafiltration of blood sera indicated $^{67}$Ga binding to non-ultrafiltrable components in serum. $^{67}$Ga protein associations were dissociated to a greater extent by exhaustive dialysis and zone electrophoresis. Both methods showed the presence of a smaller fraction of $^{67}$Ga strongly bound to proteins. These strongly bound $^{67}$Ga protein molecules in human blood were found to be transferrin and haptoglobin.

After intravenous injection of $^{67}$Ga citrate in rats $^{67}$Ga was released from the body rather slowly. $^{67}$Ga was excreted in the urine as well as in faeces but faecal excretion of $^{67}$Ga occurred later than its pronounced excretion in the urine. A number of soft tissues and bone were assayed for $^{67}$Ga and of these, the kidneys, liver and spleen showed the highest concentration. Skeletal muscle, duodenum and lungs contained an intermediate concentration while testes, brain and bone contained the lowest concentration. During 1-5 days after $^{67}$Ga citrate injection most tissues showed a clearance, and in contrast bone, testes and the brain accumulated $^{67}$Ga.
6-6 Discussion

It is now known that gallium is present in the body in trace amounts, but its function if any is not known. Gallium has been little studied in relation to its behaviour in the body, and some of these studies were reviewed in Chapter 1. The present investigations, using carrier-free tracer doses of the radionuclide of gallium (\(^{67}\text{Ga}\) citrate), in the rat permit some explanations on the blood transport, tissue turnover and excretion of this trace element.

Following the intravenous administration of \(^{67}\text{Ga}\), it was slowly removed from the body, and this is in agreement with the findings of other workers using carrier-free \(^{72}\text{Ga}\) and \(^{67}\text{Ga}\). The slow removal of \(^{67}\text{Ga}\) was brought about by its slow excretion in the urine and faeces. Initially more \(^{67}\text{Ga}\) was excreted in the urine than in the faeces, but at later times the faecal excretion was greater than the urinary excretion.

The slow removal of gallium from the body may be due to its retention in the tissues and body fluids. The study of tissue distribution of \(^{67}\text{Ga}\) resulted in the following tissue classification related to the \(^{67}\text{Ga}\) levels in tissues and the \(^{67}\text{Ga}\) clearance and accumulation in tissues:

\[(a)\] **\(^{67}\text{Ga}\) levels in tissues:**

1. High levels - kidney, liver and spleen
2. Intermediate levels - lungs, skin, testes and bone (femur)
3. Low levels - muscle, duodenum, brain, blood and blood plasma.
(b) \( ^{67}\text{Ga} \) clearance or accumulation:

1. Rapid clearance - kidney, liver and spleen
2. Fairly constant - lungs, muscle, duodenum levels and skin
3. Accumulation - bone (femur), testes and brain.

The fairly constant levels of gallium in soft tissues such as the lungs, muscle, duodenum and skin, and gallium accumulation in the testes, brain and bone would explain the slow removal of gallium from the body. The rapid clearance of \( ^{67}\text{Ga} \) from the kidney and liver may be due to the excretion in urine and faeces. When \( ^{67}\text{Ga} \) levels in these tissues were lowered, the \( ^{67}\text{Ga} \) excretion in faeces and urine was lowered. \( ^{67}\text{Ga} \) distributes in similar amounts in the blood plasma and the formed elements of blood, in which it is associated with the erythrocytes. It is claimed from the study of Belozerov (1965) that gallium associates with the erythrocyte membrane of human erythrocytes. But in the present study \( ^{67}\text{Ga} \) was recovered in the erythrocyte cytoplasm where it may be bound to haemoglobin. In plasma, \( ^{67}\text{Ga} \) clearance was rather slow, and small amounts were detected up to 5 days. This may be due to plasma protein binding.

\( ^{67}\text{Ga} \) (as gallium citrate) behaved as a negatively charged species in zone electrophoresis at physiological pH. Similar experiments with \( ^{67}\text{Ga} \) chloride also showed that gallium migrated as negatively charged species. Therefore the gallium in gallium citrate used in the present
investigations could not have been $\text{Ga}^{3+}$, but it may have been a complex of citrate, or a hydrated form of $\text{Ga}^{3+}$. It is known that $\text{GaCl}_3$ is readily hydrolysed in aqueous solution:

$$\text{GaCl}_3 + 3\text{H}_2\text{O} \rightarrow \text{Ga(OH)}_3 + 3\text{HCl}$$

The gallium hydroxide may ionise in order that an anion is formed as follows:

$$\text{Ga(OH)}_3 \equiv \text{H}_2\text{GaO}_3 \rightleftharpoons \text{H}^+ + \text{H}_2\text{GaO}_3^-$$

This would account for the $\text{Ga}^{67}$ of gallium chloride migrating as a negatively charged species at physiological pH.

Group 3 elements form chelate complexes with dicarboxylic acids, such as tartaric acid, and tricarboxylic acids such as citric acid (Cotton & Wilkinson, 1962). Pattanaik and Pani (1961) reported that aluminium citrate behaves as a dibasic acid above pH 3.1 and therefore aluminium citrate would be negatively charged at physiological pH. It is possible that the $\text{Ga}^{67}$ citrate used in the present investigations was a gallium citrate complex, having ionisable carboxyl groups. The observed anodic migration of $\text{Ga}^{67}$ in gallium citrate at the physiological pH may be due to the net negative charge of the ionised carboxyl groups of the citrate complex.
The protein binding of $^{67}$Ga in rat blood and human blood were similar, and the binding proteins in human blood were identified. $^{67}$Ga was found to associate with a number of serum proteins, principally albumin, transferrin and haptoglobin. Trivalent metal ions such as Fe$^{3+}$, Cr$^{3+}$ and In$^{3+}$ were found to bind exclusively to transferrin at low dose levels, and it is not clear why gallium binds to albumin and haptoglobin in addition to transferrin, even though the dose level of gallium in the present study was extremely low. The fraction of gallium undialysable in saline was easily removed by phosphate and in this respect gallium resembles iron which can be removed from its binding site on the transferrin molecule by phosphate (Schade et al., 1954). Gallium binds rather strongly to haptoglobin which is not normally a metal binding globulin. This binding could be due to gallium bound to haemoglobin which is then bound by haptoglobin. Haptoglobin has a high affinity for globin, whether free or in the form of haemoglobin and with these haptoglobin forms very stable complexes (Nyman, 1959). The labelling of erythrocytes with gallium was seen to be due to gallium localised in the erythrocyte cytoplasm. This may have been due to gallium binding to haemoglobin. The high concentration of gallium in the spleen may reflect erythrocyte destruction in the spleen thus releasing haemoglobin bound gallium into the bloodstream which will be bound to haptoglobin. The haptoglobin-haemoglobin complex so formed will be rapidly removed from the blood, mainly by the liver following the normal
pathway of haemoglobin degradation. In this way gallium could finally be excreted in bile and this would account for the delayed excretion of gallium in the faeces. Although no attempts were made in the present study to examine whether $^{67}\text{Ga}$ was excreted in bile it has been reported that human bile contains $^{67}\text{Ga}$ when it is injected for tumour scanning (Nelson et al., 1972).

The majority of $^{67}\text{Ga}$ was associated with plasma proteins, an ultrafiltrable fraction was found in plasma and this fraction progressively decreased with time. This fraction may represent free gallium citrate or gallium associated with other low mol. wt. ultrafiltrable components of plasma. The existence of free gallium citrate or low mol. wt. $^{67}\text{Ga}$ labelled material could account for the gallium excretion in urine initially, which later decreased when the ultrafiltrable fraction of $^{67}\text{Ga}$ in plasma decreased. Therefore gallium excretion in urine appears to be related to the nature of the gallium in blood plasma. Although gallium albumin association are weaker, it is suggested that this fraction does not directly contribute towards renal excretion of gallium for the reason that more than a third of the gallium was found to be associated with albumin at 72 h after injection, as much as at 3 h.

Gallium deposition in two experimental tumours was examined and in these solid tumours it was found that the gallium deposition was related to the degree of vascularity. The peripheral vascularised areas contained more gallium in comparison with the necrotic
areas which are poorly vascularised. In this respect
gallium distribution resembled remarkably well the
distribution of blood-borne vital dyes to various tumour
regions in benzpyrene-induced solid tumours (similar to
the RIB5 tumour used in this study), where the well
vascularised young and small tumours showed a uniform
distribution of the dye and the older
tumours concentrated the dye in the vital zones (Goldacre
& Sylvén, 1962). Unlike the vital dyes which were removed
from the tumour within 24 h, gallium remained within the
tumour up to 72 h and was found to be intracellular.
The presence of gallium in high concentration in the tumour
cell in comparison with the extracellular medium may mean
an increased affinity of the tumour cell for gallium.
Swartzendruber et al. (1971) have shown gallium localisation
in lysosomes in tumour cells, and lysosomes bring about
intracellular digestion of high mol. wt. material ingested
from the extracellular environment. It is possible that
gallium protein complexes are taken into the tumour by
phagocytosis and the gallium retained within the cell
after digestion of the proteins. However high ⁶⁷⁷⁷Ga
concentrations in the SS81 tumour were reached within
24 h and further uptake in the tumour was low.
During this period the plasma contained ultrafiltrable
gallium and therefore it is also possible that the
ultrafiltrable fraction was responsible for gallium locali-
sation in the tumour. The tumour cell, owing to its rapid
turnover and to the pathological degeneration of its
organelles, would show extensive lysosomal activity and in this instance gallium localisation in the autophagic vacuoles would still be consistent with the findings of Swartzendruber et al. (1971). It may be possible that the initial rapid uptake of gallium was due to low mol. wt. ultrafiltrable components and the later slow uptake was due to the gallium albumin fraction.

Assuming that the findings of the tissue distribution and the gallium uptake in the experimental tumours of rats can be extrapolated to the behaviour of gallium in the human body and malignant tissue in humans, certain comments can be made on the use of $^{67}$Ga citrate as a tumour scanning agent.

Gallium citrate is suitable as a tumour localising agent, in that the initial high concentration in most soft tissue is lowered in two to three days while tumour radioactivity levels are maintained thus enabling better visualisation of the tumour. However the initial high concentration in the liver, kidneys and the spleen and the presence of gallium in urine and faeces may mean that tumours present in these organs and the organs of excretion may not be prominent in scanning, owing to the extraneous radioactivity in the non-tumour area. However sequential scanning procedures may permit visualisation of tumours in these organs and their neighbourhood, due to the rapid turnover of gallium in the normal tissue.

As the access of gallium to the tumour tissue is related to the degree of vascularity of the tumour and to the degree of cellular activity and necrosis of the
tumour gallium citrate is suitable for the imaging of those tumours with good vascularity and cellular activity. The failure to visualise tumours with $^{67}$Ga citrate may therefore be related to the age of the tumour, its morphology and cytology. Therefore the absence of a positive $^{67}$Ga citrate scan in a patient with suspected cancer is no guarantee against the absence of a tumour.
following a single i.v. injection of $^{65}$ZnCl$_2$.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time after injection</th>
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<tr>
<td></td>
<td>Minutes 10 20 40</td>
</tr>
<tr>
<td></td>
<td>Hours 1 2 4 6</td>
</tr>
<tr>
<td>Blood cells</td>
<td>0.442 0.232 0.169</td>
</tr>
<tr>
<td></td>
<td>0.146 0.137 0.121</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.141 .102 .083</td>
</tr>
<tr>
<td>Testes</td>
<td>0.093 0.097 0.099</td>
</tr>
<tr>
<td></td>
<td>0.137 0.149 0.145</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.002 .002 .003</td>
</tr>
<tr>
<td>Heart</td>
<td>0.749 0.575 0.479</td>
</tr>
<tr>
<td></td>
<td>0.436 0.558 0.486</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.059 .063 .072</td>
</tr>
<tr>
<td>Lung</td>
<td>0.746 0.597 0.569</td>
</tr>
<tr>
<td></td>
<td>0.481 0.773 0.659</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.137 .122 .142</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.191 0.148 0.130</td>
</tr>
<tr>
<td></td>
<td>0.126 0.123 0.142</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.013 .009 .007</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.998 1.230 1.201</td>
</tr>
<tr>
<td></td>
<td>1.332 1.422 1.114</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.047 .133 .103</td>
</tr>
<tr>
<td>Stomach</td>
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</tr>
<tr>
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<td>0.706 0.845 0.789</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.037 .033 .043</td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.551 1.670 1.613</td>
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<tr>
<td></td>
<td>1.345 1.921 1.634</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.053 .032 .008</td>
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<tr>
<td>Prostate</td>
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<tr>
<td></td>
<td>0.195 0.349 0.428</td>
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<tr>
<td>± s.d.</td>
<td>.022 .024 .035</td>
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<tr>
<td>Liver</td>
<td>2.854 3.257 2.568</td>
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<tr>
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<td>1.829 2.063 1.672</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.460 .136 .729</td>
</tr>
<tr>
<td>pancreas</td>
<td>1.835 2.296 2.711</td>
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<tr>
<td></td>
<td>2.919 3.666 2.659</td>
</tr>
<tr>
<td>± s.d.</td>
<td>.235 .643 .886</td>
</tr>
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<td>of rats</td>
<td>5 5 5 4 4 4</td>
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</table>

-218-
following a single i.v. injection of $^{65}$ZnCl$_2$.

<table>
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<td></td>
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<tr>
<td>blood cells</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
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</tr>
<tr>
<td>estes</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>eart</td>
<td></td>
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<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>lung</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>muscle</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>spleen</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>stomach</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>duodenum</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>prostate</td>
<td></td>
</tr>
<tr>
<td>± s.d.</td>
<td></td>
</tr>
<tr>
<td>kidney</td>
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<tr>
<td>± s.d.</td>
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<tr>
<td>pancreas</td>
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<td>o. of rats</td>
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</tbody>
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THE BEHAVIOUR OF TRACER GALLIUM-67 TOWARDS SERUM PROTEINS

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(Received February 22, 1972)

SUMMARY

When carrier free gallium-67 is administered i.v. in citrate form for tumour scanning, radioactivity remaining in serum after three hours cannot be removed by ultrafiltration. Nearly a third of serum $^{67}$Ga remains undialysable and indicates the formation of strong protein–Ga complexes. By a cross electro-immuno-diffusion technique these proteins were identified as transferrin and haptoglobin. Gamma globulins do not show any detectable interactions at this dose level.

INTRODUCTION

In recent clinical trials radioactive $^{67}$Ga has been shown to localise in a wide variety of soft tissue tumours, including some deposits which escaped detection by other clinical methods$^{1-3}$. These studies also reveal a high success rate in detecting certain types of tumours. It appears that if the uptake of $^{67}$Ga in tumours can be augmented, it can play a useful clinical role as a diagnostic agent. A systematic survey into the mode of transport and the mechanism responsible for cellular uptake of gallium appears to be essential in order to attempt such manipulations.

This paper reports the aspect of plasma protein gallium interactions, and we consider that it might be of value in investigating further the behaviour of gallium in the body.

METHODS AND RESULTS

Carrier free $^{67}$Ga in citrate form was prepared in the M.R.C. Cyclotron at the Hammersmith Hospital$^4$. The patients were injected with 2.5 mCi of $^{67}$Ga-citrate intravenously as a bolus for tumour scanning$^5$. Blood samples withdrawn at 3, 24 and 72 h after gallium injection were allowed to clot in sterile tubes at 37$^\circ$ and serum was
taken for the following studies. Radioactivity of the samples and the preparations was counted in an automatic Gamma-Spectrometer (Nuclear Enterprises Ltd.).

(1) Ultrafiltration of serum samples obtained at 3, 24 and 72 h to investigate protein binding. Three ml fractions of serum were poured into Visking cellulose tubing (Scientific Instrument Centre, London), cleaned in deionised water prior to use. This was placed in a small Toribara tube and centrifuged at 2000 g for 0.5 h at 4°C and a known quantity of ultrafiltrate was counted for radioactivity. 67Ga-citrate diluted with 0.9% saline was used as a control.

In a separate experiment, binding by pure human serum albumin was investigated by mixing a 4% solution of HSA (Lister Institute, London) with 67Ga-citrate, maintaining a specific activity of 1 μCi/ml at a citrate concentration of 0.1 mM (in vivo concentration following injection). The mixture was incubated with gentle agitation at 37°C for 3 h prior to ultrafiltration. The % binding was calculated as follows:

\[
\% \text{ Binding} = \frac{A_s - A_f}{A_s} \times 100
\]

\(A_s = \) Radioactivity/ml serum; \(A_f = \) Radioactivity/ml ultrafiltrate.

Since free 67Ga (in saline controls) can be removed by ultrafiltration, this technique can be used to investigate the degree of binding of gallium by molecules retained by the membrane. Results of a typical experiment (Table I) show an increasing degree of binding to serum proteins at the time intervals studied. The binding figure for pure HSA (35%) indicates that nonalbumin proteins in serum play an important role in gallium binding. Controls failed to give the anticipated figure of zero owing to binding of gallium to the cellulose membrane but the other figures were not corrected in view of this (average 15% binding of the control). Ultrafiltration at 4°C and 37°C gave similar results.

(2) Exhaustive dialysis of 3, 24, and 72 h serum samples. For dialysis Visking bags containing 1 ml fractions of serum were secured in a Feinstein type rotary dialyser containing 1 l of 0.9% sodium chloride solution at pH 7.2 (Allen & Hanburys Ltd., London). Dialysis was continued for 24 h at 4°C.

In another study the effect of PO4 3− was examined by dialysis against a 0.06 M phosphate buffer; pH 7.4 at 4°C. 67Ga in saline solutions were used as controls.

\[
\% \text{ Binding} = \frac{\text{Radioactivity/ml of dialysed serum}}{\text{Radioactivity/ml prior to dialysis}} \times 100
\]

* Mean of six determinations ± S.D.

** Table I. **

<table>
<thead>
<tr>
<th>Sample</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h serum</td>
<td>85 ± 6.7*</td>
</tr>
<tr>
<td>24 h serum</td>
<td>97 ± 3.2</td>
</tr>
<tr>
<td>72 h serum</td>
<td>99 ± 1.9</td>
</tr>
<tr>
<td>HSA</td>
<td>35 ± 2.4</td>
</tr>
<tr>
<td>Controls</td>
<td>15 ± 2.2</td>
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</table>

TABLE I GALLIUM BINDING BY BLOOD SERA AS DETERMINED BY ULTRAFLTRATION AT 4°C

Whereas the controls showed a complete loss of activity, all serum samples retained approx. 30% of $^{67}$Ga (Table II). This fraction cannot be removed by prolongation of dialysis up to 72 h. PO$_4^{3-}$ brought about a complete loss of $^{67}$Ga from serum.

### Table II

<table>
<thead>
<tr>
<th>Dialysing medium</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>29.8 ± 2.5</td>
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<tr>
<td>Phosphate buffer</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
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</tbody>
</table>

(3) Cellulose acetate membrane electrophoresis; study with 3 h and 24 h serum samples. Zone electrophoresis of serum samples and gallium citrate was conducted on cellulose acetate membrane (CAM) in a refrigerator at 4°. A tris-barbitone sodium barbitone buffer at pH 8.4, ionic strength 0.05 was used. Approx. 20 V/cm strip length potential was applied for 0.5 h to 6.0 h. At the end of the separation membranes were dried in a horizontal position in air and cut into 0.5 cm pieces across the length for radioactivity counting. Parallel strips were stained with Ponceau-S and scanned for proteins.

$^{67}$Ga under the experimental conditions behaves as an anode-seeking species. For convenience a 2 h separation is shown (Fig. 1). After a 6 h separation no radioactivity could be detected on a 20 cm long CAM strip. However, when serum containing $^{67}$Ga is electrophoresed, a significant delay in $^{67}$Ga migration is noted (Fig. 2) and free $^{67}$Ga in control strips always migrated beyond the pre-albumin zone. When electrophoresis was continued for longer than one hour $^{67}$Ga in the albumin zone and some $^{67}$Ga in globulin zones can be dissociated leaving some radioactivity in the β globulin zone (Fig. 3). In fact dialysed sera show a $^{67}$Ga peak only at the β-zone.

(4) Visualisation of strongly bound gallium-protein complexes by cross-electroimmunodiffusion technique. The technique of Clarke and Freeman was used with some modifications in order to effect speedier separation to obtain satisfactory autoradiographs.

A bed of 1% agarose gel was prepared by pouring a mixture containing 3 ml of 2% agarose (Paines and Byrne Ltd. London) and 3 ml of 0.04 M barbitone buffer on a 5 x 5 cm clean glass plate. A hole was punched half a centimeter from one corner on the diagonal line. Ten µl of 3 h serum was added to the hole soon after collection and electrophoresed at 24 V/cm in 0.02 M barbitone buffer at 4°C in a refrigerator. After 45 min excess gel was removed leaving a 1-cm wide strip containing the proteins. Four ml of 1% gel prepared as before, containing 0.2 ml of antiserum (Hoechst Pharmaceutical, London) was poured to complete the gel-bed once again. Once the gel had set a potential of 8 V/cm was applied at right angles to the first separation for 8 h.
The excess antiserum remaining in the plate was removed by 4 successive washes in saline at 37°C and the saline was removed by washing in warm de-ionised water over a total period of 2 h. The agar bed was then dried to a thin film under a current of warm air. Radioactivity was located by autoradiography on Kodak A.R.50 film. After three days of exposure, films were developed for 10 min in Kodak D-19 developer. Ponceau-S was used to stain proteins.

Gallium-citrate controls were electrophoresed similarly.

Free 67Ga in controls moved to the very edge of the gel towards the respective anodes. All serum samples taken at 3 h show two distinctly labelled peaks (Fig. 4). These peaks were identified under similar conditions using specific antisera as transferrin and haptoglobin.

DISCUSSION

Hartman and Hayes8 working with an in vitro system demonstrated that 67Ga binding components in serum are proteins. These workers indicate that 67Ga-protein interactions at low 67Ga levels were relatively insensitive to pH changes when the pH was greater than 6.5.

Our study was designed to evaluate the nature of this binding and to identify the 67Ga-proteins complexes in serum, when carrier free 67Ga is administered i.v. in quantities normally used for clinical scanning.

The ultrafiltration data shows that 67Ga in the blood remains associated with serum proteins. Of this nearly 70% appears to be loosely associated with albumin and some globulins. This fraction can be easily removed by dialysis or zone electrophoresis. The undialysable fraction can be dissociated by PO4³⁻. In this respect 67Ga resembles iron which can be removed from its binding site in transferrin by PO4³⁻. In addition to transferrin, a haptoglobin peak can also be visualised by immuno-electrophoretic autoradiography. Other trivalent metal ions Fe, In, Cr, are known to bind exclusively to transferrin at low dose levels8,9, but gallium differs from these in that it possesses a non-specific protein binding ability.

CONCLUSION

The fact that strong associations between 67Ga and transferrin exist (similar to Fe) does not justify the assumption that 67Ga transport is exclusively brought about by this protein. Whether both albumin and transferrin serve as 67Ga carrier proteins to sites of clinical interest remains to be elucidated.

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Investigations with Radioactive Zinc Regarding its Usefulness for Pancreatic Scanning

S. Gunasekera and M. A. Chaudhri

Summary

In order to assess the usefulness of short-lived zinc isotopes like $^{62}$Zn (9.3 h) and $^{69m}$Zn (13.9 h) for pancreatic scanning, we have studied the distribution of carrier-free $^{65}$Zn (in a chloride form) in various organs of rats from 10 min to 10 days after intravenous injection. A maximum ratio of only about 1.5 between the uptake (% dose/g) in the pancreas to that in the liver was obtained at 2 hours after the injection. Attempts to enhance this ratio by starving the rats, injecting them with Tolbutamide and Diazoxide and feeding a high protein diet failed. However, when starved rats were treated with Diazoxide only and injected with a mixture (complex) of $^{65}$ZnCl$_2$ and Alpha-amino-isobutyric-acid the pancreatic uptake went up by a factor of five (6.5%/g) at 20 hours after injection. The pancreas to liver ratio of as much as almost 3 : 1 was obtained at 24 hours. This concentration ratio combined with a fairly low blood background at that time (less than 0.1%/g) may be good enough for pancreatic scanning using the well-known double-isotope and computer-subtraction techniques.

The main advantage of $^{62}$Zn and $^{69m}$Zn would perhaps lie in the fact that for the same count rate over the pancreas the radiation dose to the patient would be much smaller than with $^{75}$Se Selenomethionine (5 rads to kidneys for $^{62}$Zn as compared to 15 rads for $^{75}$Se). Moreover, the gamma energies from $^{62}$Zn and $^{69m}$Zn (510 and 440 keV respectively) being similar to the one from $^{198}$Au (410 keV) would make these isotopes much better partners in dual-isotope subtraction technique than $^{75}$Se.
Ultrafiltration for concentration and desalting of proteins in solution

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The technique of ultrafiltration by centrifugation is commonly employed to investigate plasma protein interactions with ultrafiltrable substances. The presence or absence of interactions and the degree of association can be determined as described by Toribara et al. (1957). In this laboratory the technique has been used for the study of trace element association with proteins in blood, bile and other protein solutions and for checking the efficiency of labelling of proteins with radio-nuclides. In addition, the centrifuge tube described here is used satisfactorily for concentrating macromolecules in solution prior to electrophoresis and column chromatography and as a container for Sephadex gel in centrifuge desalting.

Materials and methods
Centrifuge tubes are made from filter tubes with a disc diameter of 10 mm, porosity No. 1, length 15 cm (Scientific Supply Co. Ltd., London). These are sealed off and rounded at one end 3 cm below the disc. A small hole was made 1 cm below the disc to introduce a polythene tube of external diameter 2 mm. This is secured in position by sealing with a few layers of Parafilm (Gallenkamp Ltd., London) and warming in an oven at 45°C for 10 minutes. The other end of the polythene tube is passed through a cork. Visking cellulose tubing of flat width 10 mm (Scientific Instrument Centre Ltd., London) containing 1-3 ml of the sample is placed over the disc. The free ends are bent over and the cork is placed as shown in Figure 1. If necessary a battery of loaded centrifuge tubes can be gassed by connecting the polythene tubes crosswise to introduce the desired atmosphere.

The speed and duration of centrifugation must be determined by trial, as factors such as viscosity of the liquid often come into effect. In the author's experience with a MSE 'minor' centrifuge (MSE, London) fitted with a swing-out head, loaded with six centrifuge tubes, and centrifuged at 3,000 r.p.m. (approximate RCF value 1,500 g) an ultrafiltrate of 10% of the volume of serum is obtained in 30 minutes. The speed of the centrifuge must be increased very slowly to prevent the rupture of the cellulose membrane. The ultrafiltrate is withdrawn by unplugging the polythene tube at the Parafilm seal.

The same set up is used for concentrating macromolecules. Maximum speeds available in the centrifuge, longer spinning times and, if necessary, repetition of this step by transferring the solution into clean Visking tubing, enable concentration of the solution to desired levels. Alternatively high molecular weight substances are added (up to 3 ml) to ultra-centrifuge tubes packed with 1 g of dry Sephadex G.25 (coarse) and after 10 minutes of slow centrifugation (600 r.p.m.) followed by centrifugation at 3,700 r.p.m. (approximate RCF value 2,350 g) a concentrate of the material collects in the lower compartment within 15 minutes.

This centrifuge tube packed with 1 g of dry Sephadex G.25 (coarse) can also be used for the separation of proteins from a solution containing inorganic salts and protein preservatives. Similarly it can be used for checking the efficiency of radio active labelling of macromolecules. Sephadex gel previously swollen in water is poured into the centrifuge tube and centrifuged to remove excess water remaining outside the gel. The sample, 1/10 to 1/5 the volume of the swollen gel capacity of the tube, is introduced on top of the gel bed and allowed to permeate while at rest. After equilibration for about 10 minutes desalted proteins are obtained by centrifugation at 1,000 g.

The extent of desalting, concentration and recovery of the substance of interest depends upon parameters such as the centrifugal force applied, viscosity and the amount of sample (Emneus, (1968)), and therefore optimum operating conditions must be determined beforehand.

Miniaturisation of the ultrafiltration tube for use in the small 'bench' or 'junior' centrifuge enables the technique of ultrafiltration to be performed in a laboratory not normally equipped with a high-speed centrifuge, or when working with small volumes in any laboratory.

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