SERUM AND TISSUE PROTEIN STUDIES IN DOWN'S SYNDROME

A thesis submitted to the Faculty of Biological and Chemical Sciences for the Collaborative degree of Doctor of Philosophy in respect of work carried out at St. Lawrence's Hospital, Caterham, Surrey, and University of Surrey, Guildford, Surrey.

A.T. RUNDLE

MAY 1975
In the quarter of a century since the first observation that patients with Down's syndrome exhibited an extra chromosome (24), numerous investigators have suggested the possibility of altered protein metabolism. Most of the subsequent effects of this additional chromosome were sought in alterations in serum and tissue proteins with particular emphasis placed on the electrophoretic separation of serum proteins and the estimation of serum and tissue enzymes. Most of the claims for significant changes in enzyme levels due to the extra chromosome were later to be shown to be due to morphological changes in the tissues themselves, and apparent changes in the serum fractions to be due to the crude techniques applied.

The increasing availability of more refined techniques for the separation of serum proteins on more sophisticated support media, and the increasing use of immunological methods allows for a re-evaluation of the whole subject of possible changes in protein metabolism concomitant to the extra chromosome, and it is to this end that this thesis is directed.

In the first section, the data on sixteen specific serum and
plasma proteins in 150 subjects with Down's syndrome is com-
pared with data on a similar number of mentally retarded
control subjects. This section also contains data on
electrophoretic fractionation of serum proteins and glyco-
proteins. The structure of some 30 individual proteins were
screened using both immunoelectrophoresis and double
diffusion techniques, and the IgG fraction after purification
were subjected to papain hydrolysis and the $F_{AB}$ and $F_{C}$ fra-
gments of the Down's and control groups are compared.

In the second section, the data on the frequency distribution
of a number of serum and red cell polymorphisms and isoenzyme
systems are compared, and the genotype and phenotype
frequencies are estimated.

In the third section, an investigation into the effects of
parental age and some of the phenotype frequencies described
in the second section are described, and a relationship
between one system (haptoglobin) and the parental age at
birth of the Down's subjects is proposed.

In the fourth section, the screening of the Down's subjects
for the presence of Australia antigen (hepatitis-associated
antigen) are described, and possible relationships between
the presence of this antigen, a suggested immunological defect
and changes in proteins is considered.

In the fifth section, a comparison of the data from the first two sections with a group of 54 subjects with tuberous sclerosis is made. Both the affected groups of subjects show high mortality rates and similar patterns of infections and by comparing similarities and differences between these two groups possible changes in the Down's subjects due to selection by mortality are proposed.

In the sixth section a mathematical model is derived and applied to phenotype frequency distributions in Down's syndrome, and by its use an attempt is made to assess the value of such phenotype distributions in locating gene loci situated on the additional chromosome.

In the final section the data outlined in this thesis is summarised and in the light of the present findings suggestions are made for the continuation of this aspect of Down's syndrome.
DEDICATION

"Mit der dummheit kampfen Gotter selbst vergebens"

Schiller

This thesis is dedicated to all those who have helped me in this struggle.
I wish to acknowledge with gratitude all those persons, too numerous to name, who have collectively by their help and advice made this investigation a possibility.

I particularly wish to thank Dr. B.W. Richards of St. Lawrence's Hospital, Caterham, whose insistence on the application of scientific disciplines to the study of mental subnormality has, over the last eighteen years done so much to influence my approach to this subject, and the evidence of this influence is to be found in this thesis.

I wish to thank the following for allowing me access to their patients and for providing essential information on them:-

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INTRODUCTION

Furfuraceous idiocy was the description given by Seguin in 1846 to a specific form of mental retardation (1) which is still considered today to be the most accurate description of the physical characteristics of growth in Down's syndrome, although he considered the condition to be a sub-group of cretinism. In his classic paper of 1866 Langdon Down gave the first comprehensive description of the condition and observed that certain classes of the mentally subnormal had similar characteristics which seemed to place them within definite ethnic families (2).

In 1876, Mitchel (3) summarised his findings on 62 cases and these notes were accompanied by a perfect description of a woman with the condition made by Fraser. The following year saw enough information for Ireland to produce his monograph on Idiocy and Imbecility (4) in which he recognised Down's syndrome as a special type of mental subnormality, a suggestion not totally accepted, as shown by Shuttleworth (1886) who regarded them as unfinished children and the causation to be one of arrested development (5) (although it should be noted that this author also remarked on the high frequency of cases being the last born of a long family). The physical
stigmata now considered as typical of the syndrome were rapidly established through a series of publications which covered such features as the mouth and jaw (6), eyes (7), hands (8) and the relationship between the syndrome and heart defect (9).

The present century has seen the accumulation of a vast literature on the condition (not to mention its own peculiar mythology) establishing it as a clinical entity, and only some of the more important reviews can be mentioned.

In 1909, Shuttleworth in England presented a review of the condition (10) followed by reviews from America (11), Germany (12), Spain (13) and Engler in England in 1949 (14). It is of interesting historical note to point out that the last quoted work by Engler a quarter of a century ago was carried out in the hospital in which most of this work was done, and three of his original subjects are included in this investigation, it should also be noted that the second largest group of Down's subjects were resident at the Royal Earlswood Hospital, Redhill, which was the institution in which Langdon Down made his first observations.

The appearance of the work of Benda (15, 16) heralded the
pathological approach which can be traced to this day through monographs such as those of Donner (17) and Gustavson (18).

It had long been suspected that the condition could be associated with chromosomal aberrations (19 - 22) but at that time the cytological techniques were not adequate for the analysis of the human karyotype. With improvements of technique, Thio and Levan were able to establish the diploid number of humans to be 46 (23) in 1956, and in less than three years later Lejeune et al (24) reported the underlying chromosomal anomaly in Down's syndrome.

**Literature on Protein Metabolism in Down's Syndrome**

The presence of the additional chromosome suggested to many the possibility of alterations in protein metabolism (25 - 27) and a survey of the literature on serum proteins and the syndrome could lead to the conclusion that the information was reasonably well-documented and a high degree of agreement existed between investigators. The agreement is more illusory than real, and the range of investigations is not only superficial but limited to the extreme. In their critical review, Woodford and Bearn (28) have pointed out that many investigations were inadequately controlled, with the data on Down's subjects often being compared with normal data from different
laboratories often using alternative methods.

Although at least 70 chemical elements of the sera of Down's subjects were investigated in the decade following the chromosomal observation (29, 30) most activity was in the field of serum and tissue enzymes as a result of the first demonstration of elevated leukocyte alkaline phosphatase activity by Alter et al (26) a finding rapidly confirmed, and confirmed, and confirmed by numerous investigators (31 - 33).

The next enzyme to be the subject of intensive work and extensive claims was whole blood galactose-1-phosphate-uridyl transferase on which Brandt, in no less than three publications (35 - 37), described elevated levels, subsequently confirmed by Hsia et al (38) who found elevated whole blood levels but not in erythrocytes, the same authors failing to detect any abnormal levels in either leukocytes or erythrocytes of a number of other enzymes.

Subsequently a number of serum and tissue enzymes became implicated, including erythrocyte galactokinase (39), erythrocyte alkaline phosphatase and acetylcholinesterase (40), phosphohexokinase (41) and glutamicoxaloacetic transaminase (42) and serum pseudocholinesterase (43). Interest in enzyme levels as a method of gene location began to wane as reports based on stringent criteria began to
contradict earlier findings and finally doubt was cast even on the validity of the theoretical basis for such investigations (44).

Before the human karyotype was defined, classical genetical methods of determining gene linkage were available, but in the case of the autosomal genes it was impossible to assign a linkage group to any particular autosome. Recent advances in cytogenetics have increased the theoretical methods available for the mapping of autosomal genes, particularly where large parts of chromosomes have either been duplicated as in the case of Down's syndrome and D/D trisomy or have been deleted as in the case of myeloid leukemia (45). The procedure would involve the search for deviations in the frequency distribution of phenotypes, for example in a trisomic population, and compare this with the normal disomic population (46). Prior to the start of this investigation there was already an extensive literature on the application of the phenotype shift to the location of genes on a trisomic chromosome, but with notable exceptions (47, 48) most of the experimental work was limited to the blood group systems (49 - 58).

Since the onset of this investigation, information on the phenotype frequencies of a number of systems, both serum and
tissue, have become available, and although the literature on this field of work will be outlined in the particular section, one must quote the contributions of such investigators as Laxova in amylase heterogeneity (59), Pitt and his colleagues in serum and red cell systems (60, 61) and the work of Rittner et al on the genetic predisposition to become carriers of the hepatitis antigen (62, 63).
METHODS

Statistical Techniques

Several simple standard techniques will be used, these in­clude the mean and standard deviation (standard error), the students t-test, chi-square and correlation, but because these are so standard description will be deemed unnecessary.

Non routine methods were:-

Gene Frequencies. Suppose we select from a large population a sample of n unrelated individuals. In this population three phenotypes $G_1$, $G_2$ and $G_1 G_2$ are distinguishable, and evidence strongly suggests that these three phenotypes correspond to three genotypes $G_1 G_1$, $G_2 G_2$ and $G_1 G_2$ where $G_1$ and $G_2$ are two alleles at the same locus. If in this population (if the Hardy-Weinberg law holds) the observed numbers were $x G_1 G_1 + y G_1 G_2 + z G_2 G_2$ then the estimate for the gene frequency $p$ for gene $G_1$ and for $q$ for gene $G_2$ is given by:-

$$p = (2x + y) / 2n, \quad q = (y + 2z) / 2n$$

and the standard error is given by

$$SE_p = \sqrt{p(1-p) / 2n}$$
These equations are given by Haldane (65).

**Deviation from the Hardy-Weinberg Law (67).** If \( x, y, z, p \) and \( q \) are as defined above then the deviation from the Hardy-Weinberg law is given by (67):

\[
\chi^2 = \frac{n(y^2 - 4xz)^2}{(2x + y)^2 (y + 2z)^2}
\]

and the percentage excess of heterozygotes is \(-200 \left( pq - \frac{Y}{4n} \right)\) with an

\[
SE = 200 \sqrt{\frac{p^2 q^2}{n - \frac{1}{2}}}
\]

**Haldane's Log Ratio Test (65)**

If in two populations one was found to contain \( y \) heterozygotes and \( h \) homozygotes, and the other \( Y \) and \( H \) respectively, then the estimation of the log of the ratio of heterozygotes to homozygotes is given by:

\[
\text{Estimate} = \ln \left( \frac{(2y + 1)(2H + 1)}{(2Y + 1)(2h + 1)} \right)
\]

where \( \ln \) is the natural log.

with a \( SE = \frac{(h + y)(H + Y)}{Nn(N + n)} \) where \( N \) and \( n \) are the two population sizes.
Arcsine Transformation

The term arcsine is synonymous for the inverse sine or sine $^{-1}$, and is a transformation particularly appropriate to percentages or proportions such as will be used in Section 3. The arcsine transforms used in this investigation were obtained from the tables of Moesteller and Youtz (66).

Linear Regression Statistics and G-Statistic

The equations of the linear regression, the estimate and the SE of the coefficient of regression and the G-statistic were all carried out as described by Sokal and Rohlf (63). Occasionally the G-statistic was chosen rather than the chi-square (which is similarly distributed) as it overcomes the problems of small numbers within cells. This G-statistic is given by:-

$$G = 2 \left( \sum f \ln f + N \ln N - \sum r \ln r \right)$$

where $f$ and $r$ are the cell frequencies and the row + column frequencies respectively and $N$ is the sum of row and column frequencies.
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SECTION I

QUANTITATIVE AND QUALITATIVE STUDIES

ON SERUM AND PLASMA PROTEINS
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Review of the literature on serum proteins in Down's syndrome

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<td><strong>γ-globulin</strong></td>
<td>Griffiths et al. (1963)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pritham et al. (1963)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Carver and Welton (1961)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipoprotein</strong></td>
<td>Benda and Mann (1955)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nelson (1961)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Simon et al. (1954)</td>
<td></td>
<td></td>
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<tr>
<td><strong>IgA</strong></td>
<td>Adinolfi et al. (1967)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kaldor and Pitt (1971)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IgG</strong></td>
<td>Greene et al. (1968)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Griffiths et al. (1969)</td>
<td></td>
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<td><strong>IgM</strong></td>
<td>Greene et al. (1968)</td>
<td></td>
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<td></td>
<td>Griffiths et al. (1969)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Sutmick et al. (1959)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>IgD</strong></td>
<td>Rundle et al. (1971)</td>
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Many attempts have been made to demonstrate qualitative changes in Down's syndrome by electrophoresis (1-3), chromatography (4), ultracentrifugation (4) and other analytical methods (5, 6). An increase in gamma globulins and a concomitant decrease in albumin in institutionalised Down's subjects have been reported from many laboratories (2, 7-12) although there have been exceptions, normal albumin levels (1, 2, 13) and normal gamma globulin levels (1, 4, 15). The relevance of these findings to the intrinsic defect in Down's syndrome are questionable as they are not found in non-institutional Down's subjects (4).

Quantitative studies on serum proteins have, in the main, been concentrated on the immunoglobulins and as was shown in Table 1, are somewhat contradictory. A particularly elegant study (14) by Rowe et al in a series of immunoelectrophoretic studies of the serum proteins linked with adsorption or immunoidentity techniques failed to demonstrate any extra or missing bands but revealed concentration differences in the albumin, alpha lipoproteins, gamma globulins, and the oxidase activity of ceruloplasmin higher than normal subjects but not higher than institutionalised...
Section 1

non-Down's subjects. The increasing availability of commercial plates for the estimation of antigenically identifiable proteins by radial diffusion (12, 16) now allows for 19 proteins to be estimated, a number in excess of any hitherto possible, and this together with the increasing sensitivity of methods such as electrophoresis on newer support materials, immunophoresis, counterelectrophoresis, microdouble diffusion techniques and rocket electrophoresis should advance the study of serum and plasma proteins in Down's syndrome considerably and perhaps eliminate some of the conflicting evidence in the literature.
Section 1

METHODS

**Cellulose Acetate Electrophoresis.** Routine electrophoresis on cellulose acetate membrane was carried out on all the Down's and control subjects. The method selected was that developed by Millepore in their Clinical Electrophoresis system (Millepore: Bedford Mass.) in which the separation is carried out on support strips either 3" x 1" or 4" x 4½" in which the cellulose acetate is bonded to a semirigid mylar support. The sera are applied to the strip by means of a series of applicators consisting of two parallel welded strips which are separated to form a capillary space which will deliver 0.25 µl ± 0.05 µl. The serum proteins were separated in barbital buffer pH 0.86 (µ = 0.075) for 20 minutes at 100 volts. The strips were air dried, stained with Ponceau S dye for 10 minutes, destained in three changes of ethyl acetate/glacial acetic acid (30 : 70) and heated at 37° to dry. The cleared strips were scanned in a Phoroscope densitometer at 520 nm. The serum glycoproteins were separated in a like manner, stained with the periodic acid/Schiff technique, washed three times in 5% sodium metabisulphite, followed by two washes in tap water. After clearing in the ethyl acetate/glacial acetic the strips were scanned at 560 nm.
Fig. 1. Typical Serum Protein Fractionations Following Electrophoresis on Cellulose Acetate.

Immunophoresis. Immunophoresis was used for the qualitative screening of serum proteins. The technique adopted was basically that described in the Gellman handbook (Gellman Instrument Limited Ann Arbor : Michigan). The separations were carried out on agarose strips (7.6 x 2.5 cm) in agarose gel (1% in barbitone : sodium barbiturate buffer pH 8.8). A standard well of 1.5mm and a trough width of 1mm was adopted with a diffusion distance of 2.1mm. Electrophoresis was carried out at room temperature for two hours at 3 - 5mA per frame of six slides. Diffusion against either whole human or
specific antisera was allowed to proceed over night (16 hours), and the resulting precipitation bands located with naphthalene black (for the lipoproteins 0.5% Oil red 0 in 50% ethanol had to be used owing to the faintness of the bands, this staining technique required at least two hours for optimum staining). Fig. 2 below shows the typical separation obtained by this technique.

Fig. 2. Immunophoretic Separation of Three Down's Syndrome Sera (Whole Human Antisera).

**B - Lipoprotein (Low-Density Lipoprotein).** The low density lipoproteins were estimated using a commercially prepared pack (Boehringer Limited: London). This method involves the precipitation of the lipoprotein with a calcium chloride/heparin mixture (25nM CaCl$_2$ : 0.0125% heparin : 3.8nM NaCl), the subsequent solution of the protein precipitate in conc. sulphuric acid and the estimation of the lipoprotein
as cholesterol at 580 nm with a cholesterol reagent (50mM dimethyl sulphonate : 7.0 M acetic acid : 6.5 M acetic anhydride).

**Serum Glycoprotein Estimations**

1. **Total Glycoprotein.** Two methods were adopted to estimate the total glycoprotein, both involved an initial precipitation with perchloric acid and the subsequent precipitation of the glycoprotein with ortho-phosphoric acid (20) and their estimation either as tyrosine or carbohydrate.

**Precipitation.** In a 25ml conical flask, 0.8ml of serum was diluted with 7.2ml water, and 8.0ml 1.2% perchloric acid was added dropwise with gentle shaking and the precipitation mixture allowed to stand at room temperature for ten minutes, after which it was filtered through Whatman No. 42 filter paper (5.5cm diameter). This procedure must be performed with great care to ensure reproducibility of results as some co-precipitation of the glycoprotein is bound to occur, and the extent of this depends on the amount of mixing, precipitation time, filtration etc. (21).
Estimation As Tyrosine. To 2.0ml of the above filtrate, 0.4ml 5% phosphotungstic acid in 2N HCl were added, after mixing and allowing to stand for 10 minutes the precipitate was spun for 20 minutes at 2500 r.p.m. and washed with 1.5ml of the phosphotungstate. The precipitate was taken up in 0.6ml 15% sodium carbonate and 0.8ml water added. A tyrosine standard was prepared (100mg in 100ml 0.1 N HCl, diluted for use 1:25 with 0.1 N HCl) and a blank of the 15% sodium carbonate was prepared. To each of these three solutions 0.3ml Folin and Ciocalteau reagent were added and this reaction mixture made up to 2ml with water. After mixing, the tubes were incubated at 37° for 20 minutes and read at 700nm (1cm light path).

Total glycoprotein (as tyrosine) = \frac{O.D. \text{ Test} - O.D. \text{ Blank} \times 8\text{mg/100ml}}{O.D. \text{ Standard} - O.D. \text{ Blank}}

Estimation as Carbohydrate. To 2ml of filtrate, 0.4ml phosphotungstate was added as above, allowed to stand 5 minutes, spun as above, washed with 1.5ml ethanol, dissolved in 0.3ml 0.1 N sodium hydroxide, and after complete solution 1.7ml water added. A blank tube with 1.7ml water, 0.3ml 0.1 N NaOH and a similar standard containing 25\mu l of a
carbohydrate solution (50mg each of mannose, and galactose in 100ml water) was prepared. All three tubes were cooled in ice for 15 minutes before adding 4.0ml of a 2% orcinol solution. After shaking, the tubes were returned to the ice bath for 15 minutes, after which time they were heated at \(80^\circ \pm 0.5^\circ\) for 20 minutes and the resultant colour read at 505nm.

\[
\text{Total glycoprotein (as carbohydrate)} = \frac{\text{O.D. Test} - \text{O.D. Blank}}{\text{O.D. Standard} - \text{O.D. Blank}} \times \frac{25\text{mg}}{100\text{ml}}
\]

2. Serum Hexosamine. The total serum hexosamine was estimated by the method described by Weiden (21). To 0.2ml serum, 1.0ml N HCl were added and heated at 100$^\circ$ for 6 hours under an air condenser. After cooling the contents were adjusted to pH 6.5 with 2N NaOH and made up to 20ml. The contents were filtered through Whatman No. 42 as above, and to 1.0ml filtrate in an 8 x \(\frac{3}{8}\)" tube 1.0ml acetyl acetone were added, the walls of the tube being carefully washed down with 1.0ml water. The tubes were loosely stoppered and heated at 100$^\circ$ for 15 minutes before rapidly cooling in ice. 9.2ml Ehrlich's solution (1.5g in 75ml 95% ethanol) and 25ml conc HCl were added and the solution made up to 500ml with 95%
ethanol. After standing in the dark for 30 minutes the
colour was read at 515nm, and the concentration of hexosamine
was determined with reference to a standard curve prepared
from glucosamine HCl (20 - 140 mg/100ml).

3. Non-Glucosamine Polysaccharides (22). 0.2ml diluted
serum (1:2 with isotonic saline) was added dropwise onto 10ml
ethanol. The precipitate removed by centrifuging and washed
with a further 10ml ethanol. To the precipitate 1.0ml cold
1% aq. tryptophane was added with shaking and heated at 100°
for 20 minutes. After cooling in ice, and allowing the
solution to return to room temperature the mixture was read
at 500nm, and the concentration of the non-glucosamine
polysaccharides determined with reference to a standard curve
prepared from equiconcentrations of d-galactose and d-mannose
(0.02 - 0.16mg).

4. Sialic Acid. 2ml serum precipitated with 10ml 5% trich-
chloracetic and the tubes refluxed at 100° for 20 minutes.
After cooling and filtering, in a tube 18 x 150mm 3.0ml of
diphenylamine reagent (90ml glac acetic : 10ml conc sulphuric)
was added to 1.5ml filtrate and heated at 100° for 30 minutes.
After cooling in ice the reaction mixture was read at 505nm,
against a reagent blank and a serum blank.

**Estimation of 17 Serum and 3 Plasma Proteins by Radial Diffusion**

The proteins were estimated on commercially prepared plates (Behring:Marberg) which consists of 12 application wells containing 5 µl punched in an agarose gel containing standardised concentrations of specific antisera. The proteins thus estimated, their dilutions and running times are indicated in Table 2 below. The wells were filled with the diluted sera using 10 µl syringes together with three concentrations of the protein per plate. After diffusion the precipitation rings were measured to 0.1mm in a special viewer and the unknown diameters compared to the log. of the three standards.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. Range of Plate (mg/%)</th>
<th>Dilution</th>
<th>Running Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_1 )-acid glycoprotein</td>
<td>10 - 20</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>( \alpha_2 )-HS-glycoprotein</td>
<td>6 - 80</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>( \alpha_2 )-macroglobulin</td>
<td>20 - 230</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>( \beta_1 )-A-globulin</td>
<td>10 - 120</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>10 - 120</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>( \beta_2 )-glycoprotein</td>
<td>3 - 30</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
</tbody>
</table>
### Section 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. Range of Plate (mg/%)</th>
<th>Dilution</th>
<th>Running Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haptoglobin</td>
<td>20 - 260</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Gc Protein</td>
<td>5 - 40</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>3 - 40</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Transferrin</td>
<td>6 - 75</td>
<td>1 : 10</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>$\alpha_1$-antitrypsin</td>
<td>5 - 65</td>
<td>1 : 10</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>$\alpha_2$-antithrombin</td>
<td>3 - 30</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>$\beta_1$-E-globulin</td>
<td>3 - 20</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>3 - 30</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>25 - 250</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>2 - 12</td>
<td>1 : 2</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>IgA</td>
<td>25 - 350</td>
<td>undil.</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>IgG</td>
<td>15 - 200</td>
<td>1 : 10</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>IgM</td>
<td>30 - 400</td>
<td>undil.</td>
<td>24 hrs.</td>
</tr>
<tr>
<td>IgD</td>
<td>2 - 30</td>
<td>undil.</td>
<td>24 hrs.</td>
</tr>
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</table>

In order to obtain an accurate estimation of haptoglobin by radial diffusion it is essential to know the phenotype as equiconcentrations of the three haptoglobin types migrate at differing rates in agar. The method for the phenotyping the sera is given in Section three.
Ouchterlony Micro-Double Diffusion

Double diffusion was carried out on microscope slides in 1% agar in Gelman high-resolution buffer. The central well (antiserum) was surrounded by six sera wells at a diffusion distance of 5mm (well diameters 3mm). Undiluted sera were allowed to diffuse against the antisera at room temperature for 16 hours, and the resulting precipitation lines examined for crossing of the arcs or for spur formation. In addition to the proteins in Table 2 the following proteins were screened:

- Albumin
- $\lambda_1$-B-glycoprotein
- $\lambda_1$-T-glycoprotein
- $\alpha$- lipoprotein
- $\beta$-lipoprotein
- $\beta_2$-glycoprotein
- $\beta_2$-glycoprotein
- $\beta_2$-glycoprotein
- Cholinesterase
- C'-esterase inhibitor
- Inter-$\lambda$- trypsin inhibitor
- Uromucoid
- $\alpha_1$-antichymotrypsin

The specific antisera for this screening was obtained from Behring: Marberg.
Section 1

IgG Hydrolysis by Papain and Examination of F\textsubscript{AB} and F\textsubscript{C} Fragments.

The papain hydrolysis method is basically that described by Porter (24), the purification of the IgG fraction is a mixture of reported techniques described by Schultz and Heremans (25).

A 4M solution of ammonium sulphate was added to pooled sera from Down's subjects to a final concentration of 37.5% in a cold room with constant stirring. The resulting precipitate was centrifuged at 5° at 18,000 r.p.m. The supernatant was removed and the precipitate washed with cold 37.5% ammonium sulphate. The precipitate was taken up in phosphate buffer pH 7.0 and checked by immunophoresis against monospecific IgG antisera and found to be predominantly IgG. Starch gel electrophoresis revealed some contamination with albumin and a purification step was introduced. Aluminium chloride (0.1 M) was added to the IgG solution in a 1:1 ratio at 0° (25) and the pH reduced to 4.7 with 0.1 N NaOH. After 5 hours the resulting precipitate was removed at 0° and discarded, the purified IgG precipitated by adding 95% ethanol at -6° to a final concentration of 25%. The precipitated IgG washed with cold 25% ethanol and after freeze drying
could be taken up in the phosphate buffer as above. Electrophoresis and immunophoresis could detect no appreciable contamination. Approximately 1g of the IgG was dissolved in the hydrolysis buffer (0.1 M phosphate : 0.01 M cystein : 2 mM EDTA). To this was added 0.1 ml papain suspension (0.05 acetate buffer pH 4.5) and the volume adjusted to 20ml. Hydrolysis was allowed to proceed at room temperature overnight, the reaction mixture dialysed against several changes of water for 48 hours and freeze dried. The resulting precipitate taken up in phosphate buffer pH 7.0 and examined by immunophoreses and double diffusion against whole human antisera and against antisera specific to $F_{AB}$ and $F_{C}$ fragments.
## RESULTS

### Table 3

Densiometric data on the serum proteins and glycoproteins of subjects with Down's syndrome and institutionalised control subjects, after cellulose acetate electrophoresis.

<table>
<thead>
<tr>
<th>Test</th>
<th>Down's Syndrome</th>
<th>Controls</th>
<th>t-test</th>
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<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>Serum proteins (g/100ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>64</td>
<td>6.10</td>
<td>2.04</td>
</tr>
<tr>
<td>Albumin</td>
<td>64</td>
<td>2.80</td>
<td>0.06</td>
</tr>
<tr>
<td>α1-globulin</td>
<td>64</td>
<td>0.29</td>
<td>0.20</td>
</tr>
<tr>
<td>α2-globulin</td>
<td>64</td>
<td>0.74</td>
<td>0.21</td>
</tr>
<tr>
<td>β-globulin</td>
<td>64</td>
<td>0.93</td>
<td>0.26</td>
</tr>
<tr>
<td>γ-globulin</td>
<td>64</td>
<td>1.48</td>
<td>0.45</td>
</tr>
<tr>
<td>Serum glycoproteins (% of total)</td>
<td>153</td>
<td>14.1</td>
<td>5.8</td>
</tr>
<tr>
<td>α1-glycoprotein</td>
<td>153</td>
<td>14.1</td>
<td>5.8</td>
</tr>
<tr>
<td>α2-glycoprotein</td>
<td>153</td>
<td>40.3</td>
<td>7.4</td>
</tr>
<tr>
<td>β-glycoprotein</td>
<td>153</td>
<td>26.0</td>
<td>4.0</td>
</tr>
<tr>
<td>γ-glycoprotein</td>
<td>153</td>
<td>18.9</td>
<td>6.2</td>
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**p < 0.01

### Table 4

Down's Group, Total Glycoprotein as

<table>
<thead>
<tr>
<th>Tyrosine (mg/100ml)</th>
<th>Carbohydrate (mg/100ml)</th>
<th>Hexosamine (mg/100ml)</th>
<th>Polysaccharide (mg/100ml)</th>
<th>Sialic Acid (Optical Dons.)</th>
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</thead>
<tbody>
<tr>
<td>5.26</td>
<td>19.2</td>
<td>84.2</td>
<td>108</td>
<td>0.091</td>
</tr>
<tr>
<td>6.31</td>
<td>21.5</td>
<td>124.8</td>
<td>152</td>
<td>0.108</td>
</tr>
<tr>
<td>4.01</td>
<td>21.6</td>
<td>118.7</td>
<td>94</td>
<td>0.083</td>
</tr>
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<td>3.40</td>
<td>18.9</td>
<td>122.6</td>
<td>104</td>
<td>0.069</td>
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<tr>
<td>6.36</td>
<td>22.4</td>
<td>108.1</td>
<td>92</td>
<td>0.141</td>
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<td>4.99</td>
<td>23.3</td>
<td>90.5</td>
<td>98</td>
<td>0.122</td>
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### Section 1

<table>
<thead>
<tr>
<th>Down's Group Total Glycoprotein as</th>
<th>Down's Group</th>
<th>Non-Glucosamine</th>
<th>Sialic Acid (Optical Dens.)</th>
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<tr>
<td>Tyrosine Carbohydrate (mg/100ml)</td>
<td>Hexosamine (mg/100ml)</td>
<td>Polysaccharide (mg/100ml)</td>
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<tr>
<td>3.10</td>
<td>82.2</td>
<td>106</td>
<td>0.065</td>
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<td>5.44</td>
<td>87.9</td>
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<tr>
<td>9.82</td>
<td>94.4</td>
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<tr>
<td>4.64</td>
<td>121.1</td>
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<td>5.82</td>
<td>130.6</td>
<td>124</td>
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<td>8.26</td>
<td>89.3</td>
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<td>0.131</td>
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<tr>
<td>3.91</td>
<td>95.8</td>
<td>138</td>
<td>0.081</td>
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<tr>
<td>7.15</td>
<td>115.7</td>
<td>106</td>
<td>0.130</td>
</tr>
<tr>
<td>5.50</td>
<td>111.2</td>
<td>102</td>
<td>0.099</td>
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<tr>
<td>3.13</td>
<td>119.2</td>
<td>120</td>
<td>0.133</td>
</tr>
<tr>
<td>7.28</td>
<td>93</td>
<td>115</td>
<td>0.126</td>
</tr>
<tr>
<td><strong>Mean</strong> 5.48</td>
<td><strong>104.2</strong></td>
<td><strong>110.5</strong></td>
<td><strong>0.103</strong></td>
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<tr>
<td><strong>SD</strong> 1.77</td>
<td><strong>3.5</strong></td>
<td><strong>15.6</strong></td>
<td><strong>0.023</strong></td>
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<table>
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<tr>
<th>Controls (n = 19)</th>
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<tbody>
<tr>
<td>Mean 4.9</td>
<td>101.8</td>
<td>118.3</td>
<td>0.117</td>
</tr>
<tr>
<td><strong>SD</strong> 1.53</td>
<td><strong>4.0</strong></td>
<td><strong>9.6</strong></td>
<td><strong>0.015</strong></td>
</tr>
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### T-Test Between Groups:

|                  | 1.1        | 2.0        | 0.62 | 1.65 | 2.18 |

### Reported Normal Ranges

<table>
<thead>
<tr>
<th></th>
<th>Mean 3.3</th>
<th>21.8</th>
<th>101.5</th>
<th>114</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SD</strong></td>
<td>1.0</td>
<td>6.3</td>
<td>11.0</td>
<td>3.2</td>
<td>-</td>
</tr>
</tbody>
</table>
Section 1

Electrophoretic data on the serum proteins and glycoproteins are shown in Table 3. The significant reduction of total protein in the Down's group was surprising as one of the most consistent findings of other surveys is a normal total protein level (see Table 1). The reduction in this total level is mainly due to the reduction in albumin levels, but significant reductions of the $\alpha_2$- and $\beta$-globulins also make some contribution. The reduction in these latter two are in agreement with other surveys (1, 19). No significant reduction in gamma globulins is in evidence, which confirms several other investigations (1, 4, 37). Significant reductions in the percentage contribution of the $\alpha_1$- and $\beta$-glycoproteins can also be seen in Table 3. In the less sensitive paper electrophoretic survey Nelson et al (3) also reported a decrease of the $\beta$-glycoproteins, but failed to show any changes in the $\alpha_1$-fraction. A similar finding to the reductions in these two fractions have been reported (26) but reference must be made to a report of the failure to detect any differences in the fractions when estimated as polysaccharides (4).

From the Down's group, eighteen subjects were drawn at random
and a series of quantitative tests for glycoproteins were carried out as shown in Table 4, together with the same tests carried out on 19 control subjects also drawn at random and the t-test between the means for these two groups. In no instance could any significant difference between the two means be found.

Table 5 sets out the findings on the four principal immunoglobulins in the two test groups and compares them by means of the t-test.

**Table 5**

<table>
<thead>
<tr>
<th>Immunoglobulin (mg/100 ml)</th>
<th>Down's syndrome</th>
<th>Controls</th>
<th>Normal (Särkä 1968)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>IgA</td>
<td>105</td>
<td>321.9</td>
<td>89.0</td>
</tr>
<tr>
<td>IgG</td>
<td>113</td>
<td>1520</td>
<td>522</td>
</tr>
<tr>
<td>IgM</td>
<td>119</td>
<td>119.8</td>
<td>65.5</td>
</tr>
<tr>
<td>IgD</td>
<td>90</td>
<td>7.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

From the data in Table 5 it can be seen that the IgA and IgD are significantly elevated whereas the other two immunoglobulins are within the expected range. Numerous surveys have suggested that a raised IgG level in Down's syndrome is
Section 1

common (see Table 1) but as most of these rely on a comparison with non-institutionalised controls the finding in this investigation is probably a more accurate assessment of the situation. In Section 4 of this thesis the data on the immunoglobulins will be assessed in the light of an abnormal immunological response in Down's syndrome.

<table>
<thead>
<tr>
<th>Protein Fraction (mg/100 ml)</th>
<th>Normal (Child and Margaret 1966)</th>
<th>Normal (Gibbons and Gibbons 1966)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>G.C. proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alpha-glycoproteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta-glycoproteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>transferrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferritin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total immunoglobulins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-idiotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-idiotypic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-idiotypic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total immunoglobulins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-idiotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-idiotypic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-idiotypic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In three fractions where the Down's syndrome level appears to differ significantly from the reported normal, data are given on an institutionalised control subject.

**Significant at p < 0.05.

***Significant at p < 0.01.

Table 6

Comparison of the levels of 14 specific proteins in the serum of subjects with Down's syndrome and the reported levels in normal subjects.
As no previous reports on the levels of IgD in Down's syndrome could be found, and there are several reports of a sex difference in the frequency of infections in Down's syndrome (see Section 4) the IgD data has been divided by sexes and the distribution of the data for the two sexes is shown in Fig. 3 below. The horizontal bar indicates the mean. No significant difference could be detected between the sexes ($t = 1.51$).

![Fig. 3](image)

Fig. 3 Distributions of Serum IgD Levels Between Male and Female Down's and Control Subjects.

The quantitative data on 14 serum proteins in Down's syndrome are summarised in Table 6, together with data on the control
groups for those proteins which appear to deviate from the normal reported data. Comparison with the normal reported data (27, 41) failed to reveal any significant changes in fractions other than the \( \alpha_1 \)-antitrypsin levels, \( \alpha_1 \)-acid glycoproteins, transferrin and haptoglobin. The interpretation of the apparent abnormal levels of haptoglobin are made difficult by the differing proportions of the three phenotypes found in different normal populations. Elsewhere I have shown that the three phenotypes are associated with differing levels of protein even when the changes in mobility are taken into account (28). In that publication I was able to show that the mean level of haptoglobins were:

- \( \text{Hp 1 : 1, 119.5 mg/100ml (SD 32.1)} \)
- \( \text{Hp 2 : 1 it was 208.2 mg/100ml (SD 71)} \)
- \( \text{and for Hp 2 : 2 it was 223.0 mg/100ml (SD 71)} \)

When 94 subjects were selected from the control data having the same phenotype distribution as the Down's subjects in Table 6 the total mean haptoglobin was found to be 209.3 mg/100ml (SD 92.1) which does not differ significantly from Down's syndrome. Of the other three apparently abnormal proteins in Table 6 comparison of two of them (transferrin and \( \alpha_1 \)-antitrypsin) with the institutionalised control group failed to confirm the difference. The third, the \( \alpha_1 \)-acid
glycoprotein remained elevated ($t = 7.2$, $P = 0.001$).

Although it is outside the scope of this thesis, it is interesting to note that compared with the non-institutionalised control subjects (normals) the $\alpha_1$-acid glycoprotein levels in the institutional controls are significantly reduced ($t = 2.1$, $P = 0.05$).

Table 7 summarises the levels of three plasma proteins in Down's syndrome and compares them with the control group and reported normal data. Comparison of the means by t-test failed to reveal any significant differences between the two groups in any of the three fractions.

Table 7

Levels of Three Plasma Proteins in 59 Subjects with Down's Syndrome and 61 Institutionalised Control Subjects.

<table>
<thead>
<tr>
<th>Protein Fraction</th>
<th>Down's Mean</th>
<th>SD</th>
<th>Control Mean</th>
<th>SD</th>
<th>t-test</th>
<th>Normal (27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/100ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>303.2</td>
<td>73.1</td>
<td>296.0</td>
<td>59.1</td>
<td>0.58</td>
<td>200-450</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>17.1</td>
<td>4.4</td>
<td>18.3</td>
<td>3.05</td>
<td>1.05</td>
<td>15-20</td>
</tr>
<tr>
<td>$\alpha_2$-Antithrombin</td>
<td>33.4</td>
<td>10.1</td>
<td>36.2</td>
<td>8.2</td>
<td>1.7</td>
<td>-</td>
</tr>
</tbody>
</table>

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**Section 1**

**Structural Changes.**

Comparison of the 33 proteins listed in the methods section between the Down's and Control groups, double diffusion and immunoelectrophoretic failed to show any structural differences, nor were any additional precipitation lines observed other than the transformation of the $\beta_1$-C- globulin to $\beta_1$-A-globulin which has been shown to occur spontaneously in normal sera (28). In one reported study it was suggested that there are locational differences in both the $\alpha_1$-globulins and the $\alpha_2$-lipoprotein regions (14) but using the 'short trough' technique this could not be confirmed. The Authors of that report (14) were in some doubt whether the locational differences were due to structural changes or were a concentration effect. The double diffusion method confirmed there were no measurable changes in immunological response of the proteins studied, for as Ouchterlony has pointed out, 'if the arcs fuse but neither cross nor spur the proteins are immunologically identical' (23). The above observations suggest that the locational differences referred to above are most likely due to concentration effects.

**Structure of the IgG Fraction**

There have been several suggestions on the structural change
Section 1

in the gammaglobulins in general and the IgG fraction in particular (see Table 1). The mobility of IgG from the two test groups were compared by electrophoresis and immunoelectrophoresis and no such differences could be observed.

Fig. 4

Fig. 4. Immunoelectrophoretic Patterns; Upper and Lower Well - Hydrolytic Product of Purified IgG from Down's Syndrom. Centre Well - Purified IgG from Control Sera. Upper trough - Anti-μ Serum. Lower trough Anti-μ Serum.

Fig. 4b. As in Fig. 4a but Controlhydrolysate, Centre Well Unpurified IgG.
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Pooled sera IgG from both groups was subjected to papain hydrolysis followed by immunophoresis with diffusion against antisera specific for the $F_{AB}$ and $F_C$ fragments. The results of this are shown in Fig. 4 even casual inspection shows that the increased mobility of the $F_C$ fragment that has been reported (31) could not be confirmed.
One of the major problems in assessing the value and validity of any survey on serum and plasma proteins in Down's syndrome is the difficulty is assigning any changes observed, to the syndrome per se or to the increased frequency of infections in the syndrome which itself may either be due to the condition itself or due to the whole process of institutionalisation. Although increased frequency of infection is a general finding in most surveys (8, 15, 32, 34) the risk seems to be variable in the extreme, probably due to variations in success of instituting a satisfactory regime of hygiene. It is this variation in infectious rates which probably accounts for many of the divergent views put forward by previous investigators. One good example of this was the failure to find reduced levels of albumin in a sample of Down's subjects who had only recently been admitted from home (8). As will be shown in Section 4, there is little to show that the elevation in the levels of IgA and IgD is other than the normal response to a chronic infectious situation, although it has been suggested that because the Down's subjects are so prone to infections that for this reason alone they are usually subjected to a more rigorous programme of vaccination and it may be this which contributes to the abnormal findings (8, 35, 36).
Section 1

The lack of differences between the protein levels of the two groups investigated confirms similar findings elsewhere (37) and stresses the need for careful control of the 'normal' subjects selected for comparison (38).

At first sight the results of the electrophoresis by cellulose acetate and the estimation of the individual proteins by radial diffusion appear to be contradictory, with the former suggesting a lack in the $\alpha_1$-glycoprotein whereas estimation by radial diffusion suggests the opposite. Such differences arise through the lack of sensitivity of electrophoretic methods and through non-availability of antisera to proteins known to exist in serum. The $\alpha_1$-glycoprotein band by electrophoresis is known to contain not only the $\alpha_1$-acid glycoprotein and $\alpha_1$-antitrypsin estimated by radial diffusion but also $\alpha_1$-$\beta$-glycoprotein (easily precipitable glycoprotein, Trp-$\alpha_1$-glycoprotein (tryprophane poor), $\alpha_1$-$\kappa$-glycoprotein and the post albumin 4.6 S PoS (25). It may be that the very divergence of the results obtained by the two techniques will prove more interesting than the actual data and a search for differences in these lesser fractions is indicated. One of the earliest and still most consistent findings is the elevation in IgG levels, a finding not supported by this
investigation, nor was the suggestion of structural differences in the $F_C$ fragment confirmed. However, it should be noted that the suggested $F_C$ abnormality arose during studies in neonatal Down's subjects, and there is evidence that these neonates are atypical of Down's subjects as a whole, depressed levels of IgG in newborn Down's have been reported (31) as also have abnormal immunophoretic patterns (39).
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SECTION 2

SERUM AND TISSUE POLYMORPHIC SYSTEMS.
Section 2

INTRODUCTION

Ford has defined genetic polymorphism as "the occurrence together in the same habitat of two or more discontinuous forms or phases in such proportions that the rarest of them cannot be maintained merely by recurrent mutation", (1). Although heterozygote advantage is thought to play an important part in maintaining this polymorphic balance, the roles played by specific diseases and clinical entities are only beginning to be explored. Although the search for unusual distributions of genetic markers in disease states is one approach to this problem of determining the factors which keep polymorphism in balance it is not appropriate for Down's syndrome since this condition is attended by a gross reduction in fertility.

There are, however, compensatory inducements for investigating polymorphism in Down's syndrome, among others, these include possible association with late maternal age, changes in frequencies resulting from the high mortality rate and possible association with the much-reported increased infection rates.
The earliest work on possible changes in the frequencies of polymorphic systems predate the discovery of the additional chromosome by several years, and reported extensive investigations on blood typing (ABO, MNS, Rh, P, Lewis and Kell) (2-9) occupied a number of investigators for more than half a century. Prior to the start of this investigation with the exception of blood grouping and one study on haptoglobins and transferrins (11) and a study of several systems on a very small population (10) (both surveys which have been criticised on several counts) the information on this aspect of Down's syndrome is surprisingly sparse.

This section will report on seven serum and nine polymorphic systems from red cells, and data on the phenotype frequencies, gene frequencies, deviation from the Hardy-Weinberg law, genotype and phenotype association, and the effects of age and sex on these will be adduced. The systems to be investigated will be:

**Serum Haptoglobin.** Three main forms of haptoglobin are encountered in most populations and these are due to the inheritance of two autosomal alleles Hp\(^1\) and Hp\(^2\) which in combination give rise to the three principal phenotypes
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Hp 1:1, Hp 2:1 and Hp 2:2 (12, 13). Investigation of large numbers of individuals of various ethnic origin has revealed a number of quantitative and qualitative variants, all of which have a low frequency and represent sporadic mutation of the two principal alleles (14).

Serum Group Specific Proteins. The variability of a group of alpha-2-globulins, subsequently named group specific or Gc proteins was first recognised by Hirschfield (1960) (15), and later shown to be a polymorphic system inherited as a pair of co-dominant autosomal genes giving rise to three phenotypes Gc 1:1, Gc 2:1 and Gc 2:2 (16). Although a number of variants have been described, these are very rare.

Serum $\beta_2$-Glycoprotein 1. Schultze et al (1961) (17) were able to demonstrate the presence in serum of an unknown $\beta_2$-globulin having a molecular weight of 40,000. Subsequently, Augener estimated the amount of this protein in the sera of normal adults and found a range of 23.5 ± 4.4 mg/100ml (18). Although the concentration of this protein is influenced by such external factors as age, sex, chronic liver diseases, pregnancy, etc., it has been shown to be controlled by a pair of autosomal genes giving rise to three phenotypes Bg N:N, Bg N:D and BgD:D with the BgN allele being more common (19).
Serum Leucine Naphthylamidase B-Band. Polymorphism of this enzyme (EC 3.4.1.1) is not under simple genetic control as in the above three examples. The sera of all normal subjects exhibit a band of activity, but some exhibit an additional slower running band (34) which varies in concentration from total absence to relatively high concentrations. Studies on twin and family data (7, 21) showed that this additional band (B-band) is partially under genetical control, but lack of complete concordance suggests that environmental factors may influence its presence in the serum, as can compounds such as oral contraceptives (22) and drugs (21, 23).

Serum Cholinesterase (Pseudocholinesterase). The analysis of the genetical control of the serum cholinesterases has made considerable progress in recent years, and it has become clear that there are a number of loci involved (32 - 38) with a number of phenotypes requiring inhibition techniques (Dibucain, fluoride, Ro number) for their identification. In 1963, Harris et al (56) described an additional phenotype which they termed C5 which could be characterised by an additional band of activity moving slower on starch gel than the common C4 band, and showed that in a normal British
population a frequency of 0.13 could be anticipated.

**Red Cell Adenosine Deaminase.** By means of a starch gel electrophoresis and specific staining technique Spencer et al (39) demonstrated the occurrence of three different adenosine deaminase patterns in human red cells, and family studies proved that these were due to two alleles $ADA^1$ and $ADA^2$. Some rare additional alleles have been described (40, 41).

**Red Cell Pyruvic-Glutamic Transaminase.** Glutamic-pyruvic transaminase (ALT) is one of several enzymes which exist in both cytoplasmic (soluble) form and mitochondrial (residual) form. In mature red cells only the soluble form is present (42 - 44) and genetic polymorphism of this soluble enzyme representing two alleles $ALT^1$ and $ALT^2$ have been described (44).

**Red Cell Phosphoglucomutase.** Phosphoglucomutase (PGT) is an enzyme in which three structural gene loci affecting the molecular characteristics of the different PGT isozyme components have been identified (46). Each PGT locus determines a characteristic set of two or more isozymes with none of these appearing to hybridise. A series of electrophoretic
variants attributable to the occurrence of multiple alleles of each of the three loci have been identified (47). In most tissues PGT\(^1\) locus predominates and accounts for some 90 - 95% of the total activity; the PGT\(^2\) locus isozymes account for some 5 - 10% with less than 2% being contributed by the PGT\(^3\) locus. The greater contribution of PGT\(^1\) is possibly due to the differences in relative rates of synthesis, but there is a distinct possibility that the different sets of isozymes differ markedly in specific activity. In the red cells, the PGT\(^1\) and PGT\(^2\) loci both contribute about 50% of the activity with no PGT\(^3\) products occurring possibly due to the unstable anucleate state of the cell, although some instances of 'young' red cells exhibiting weak PGT\(^3\) activity has been reported (47).

Red Cell Acid Phosphatase. Red cell acid phosphatase (AcP) is a phosphohydrolase and phosphotransferase with a low optimum pH which has so far been detected only in erythrocytes (47), differing in substrate specificity from the acid phosphatases existing in other tissues. Three principal alleles are known to exist at the red cell locus the A, B and C alleles giving rise to six recognisable phenotypes with the B allele most common and the C allele least common in British populations.
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Red Cell 6-Phosphogluconate Dehydrogenase. This enzyme system (PGD) was first shown to exhibit polymorphism in erythrocytes (48), and studies have shown the three common variants to be due to a single-locus, two allele system (49). A number of uncommon variants have been described, including the Richmond and Hackney types (50) and the Frieberg variant (51), all of which are associated with bands of the A type, the two former variants having additional slower bands, and the latter a single additional faster band.

Red Cell Adenylate Kinase. (AK) Adenylate kinase is a phosphotransferase:

\[ 2 \text{ADP} \rightarrow \text{ATP} + \text{AMP} \]

and is present in most human tissues with particularly high levels in red cells and skeletal muscles (46). Using starch gel electrophoresis it is possible to recognise three common European phenotypes due to the combination of two autosomal alleles at the AK locus (52).
Glucose-6-Phosphate Dehydrogenase. (G-6-PD). The discovery that primaquine-induced sensitivity was associated with G-6-PD deficiency in the red cell (53) led to many investigations with important results in the field of clinical medicine, genetics and anthropology. Subsequent investigations have shown many genetic variants to exist (enough to warrant a WHO publication (54)). Some of these variants are associated with normal enzyme activity and have no clinical manifestations (e.g. the A variant in Negroes) while others are associated with haemolytic disorders in the presence of external factors (fava beans, drugs and infections). Most variants are rare but occasionally a variant is found to be common to a particular population (presumably due to selective advantage), examples of these are the Mediterranean and A -deficiency types which probably owe their survival to selective advantage vis-a-vis Falciparum malaria (55).

Isocitric Dehydrogenase. NADP-dependent isocitric dehydrogenase (ICD) catalyses the oxidative decarboxylation of L-citrate to L-ketoglutarate, and like the enzyme ALT referred to above exists in most tissues in two forms a soluble and a residual form (85 - 88), and also like the ALT only exists in the soluble (cytoplasmic) form in the erythrocytes. Rare
variants of this system have been reported with electrophoretic patterns consistent with a dimeric molecular structure, with family studies suggesting that the enzymes structural locus is autosomal and distinct from the locus governing the mitochondrial forms of the enzyme (92).

**ABO Locus and Haemoglobin.** These two systems require little in the way of an introduction, the ABO locus is an internationally recognised genetic marker with reported normal frequencies in practically all conceivable populations and in most abnormal populations. Haemoglobin exists in two principal forms in the adult with a main A band and a slower migrating A3 band, additionally in some electrophoretic systems the A band can be shown to be heterogeneous being capable of splitting into an additional A2 band.
Section 2

METHODS

\( \beta_2 \)-Glycoprotein 1. The quantitative estimation of this protein is fully described in the first section of this thesis together with observed data on the two test populations. Cleve (19) suggested that individuals homozygous for the gene Bg N should be limited to those with serum levels of 16 - 30mg, the subjects heterozygous for the genes BgN and BgD showed an intermediate level of 6 - 14mg and the homozygous Bg D D less than 5mg 3100 ml. Reported normal frequencies include a number of European populations but do not include a normal English frequency (19, 56, 91), and to rectify this a sample of 381 normal healthy adult English subjects were tested and the results of this survey are in Fig. 1 below.

Slight differences were shown in the English population, and the criteria for the three phenotypes that was finally accepted were:-

- Type Bg D:D less than 4mg/100ml
- Type Bg D:N 4 - 14 mg/100ml
- Type Bg N:N Equal or greater than 15 mg/100 ml.
Serum Gc Proteins. Although a number of well documented electrophoretic methods are available for the phenotyping the Gc proteins, immunoelectrophoresis was the final method of choice, as it combined the separation by electrophoresis with the specificity of precipitating antibodies. Details of the technique are described in the first section of this thesis.

ABO Blood Groups. Grouping was carried out by
haemagglutination using commercially prepared antisera with sub-grouping of the A group not being carried out.

Starch Gel Electrophoresis. With the exceptions noted above, phenotyping was carried out on horizontal starch gels (15 x 0.6 x 15cms) in batches of 10 - 12 sera, the gels being prepared from 11% starch in various buffers. The runs were carried out in a cold room at 5° overnight at 75 - 200 volts depending on the system being used. After slicing the proteins were located using specific staining or substrate mixtures, either directly on the cut surface or incorporated in 1% agar which was poured onto the cut surface and the locating agents allowed to diffuse into the starch gel. Details of these techniques are given below:-

<table>
<thead>
<tr>
<th>Locating Agent</th>
<th>Locating Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alk. Phosphatase</td>
<td>Direct on cut surface.</td>
</tr>
<tr>
<td>100ml 0.1M tris (pH8.8) + 10 drops aq MgCl₂ + 100mg Naβ-Naphthylphosphate + 100mg Blue RR salt.</td>
<td>Heat at 37° for 5 - 10 minutes.</td>
</tr>
</tbody>
</table>
### Locating Agent

**Leucine Naphthylamidase**

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg L-leucyl-4-naphthylamide - HCl in 25 ml dimethyl formamide, diluted to 50 ml with phosphate buffer pH 7.0.</td>
<td>50 mg Black K salt in 50 ml phosphate buffer pH 7.0.</td>
</tr>
</tbody>
</table>

**Haptoglobin**

- 100 ml water + 0.2 g benzidine + 0.5 ml glacial acetic acid.
- 2 ml hydrogen peroxide (100 vols.) added before use.

**Phosphoglucomutase**

<table>
<thead>
<tr>
<th>Stain Buffer</th>
<th>Stain Buffer (cont.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ml 0.2 M tris: HCl pH 8.0 + 0.17 g Na₂ glucose-1-phosphate. 4H₂O + 2.2 mg glucose 1,6 diphosphate. 2H₂O + 10 ml buffer containing 20.3 mg MgCl₂ + 10 mg NADP + 80</td>
<td>In 75 ml 2% aqueous agar overlay and incubated at 37° until optimum bands appear.</td>
</tr>
</tbody>
</table>

### Locating Technique

**Direct**

- Develop at room temperature with soln. A for 2 hours. Replace with soln. B develop at room temperature.

- Heat at 37° until bands are optimum.
Section 2

Locating Agent

Phosphoglucomutase (contd.)

units glucose-6-phosphate dehy-
drogl. + 1mg PMS + 10mg MTT.

Made up to 50ml with water.

C5 Esterase

100mg Fast Blue RR + 10ml tris
HCl (pH 7.0) + 3% NaOAc-naphthyl
acetate + 87ml water.

Haemoglobin

As for haptoglobin above.

Adenosine Deaminase

40mg adenosine + 10mg MTT +
10mg phenazine methosulphate +
0.16 units Xanthine oxidase +
1.6 units nucleoside phosphory-
lase.

Glucose-6-Phosphate Dehydrogl.

2mg NADP + 2mg MTT + 2mg PMS +
10mg Na2 Glucose-6-phosphate in

Locating Technique

Direct at room temperature
for 30 minutes.

As for haptoglobin above.

In 100ml 2% agar in 0.025M
phosphate buffer pH 7.5.

Incubated at 37° until
optimum bands.

Soaked into filter paper and
applied direct. Incubate at
### Locating Agent

**Glucose-6-Phosphate Dehydrogenase (cont'd).**

10 ml 0.5M tris HCl pH 8.0.  

37° until optimum.

**6-Phosphogluconate Dehydrogenase.**

3 mg NADP + 6 mg MTT + 1.2 mg PMS  

In 1% agar overlay. Incubate + 30 mg Na₃-6-phospho-gluconate in at 37° until optimum.  

10.5 ml 0.5M tris HCl. Dilute to 30 ml.

### Locating Technique

**Acid Phosphatase**

0.163 g phenolphthalein diphosphate in 50 ml 0.05M citrate buffer pH 6.0.  

Soak into filter paper and apply to cut surface. Wrap in plastic film and incubate at 37° for 4 hours. Sprinkle NH₄OH on the filter paper.

**Adenylate Kinase**

6 ml 0.5M tris HCl pH 8.0 + 3.0 ml 0.1 M MgCl₂ + 60 mg glucose + 15 mg ADP + 7.5 mg NADP + 4.5 units G-6-PD + 9 units hexokinase + 7.5 mg MTT + 7.5 mg PMS.  

Soak onto filter paper apply direct incubate at 37°.
### Section 2

#### Locating Agent

**Glutamic-Pyruvic Dehydrogenase**

- 0.3564 g l-alanine + 0.0254 g α-ketoglutarate + 0.0124 g NADH₂ in 20 ml 0.1 M tris HCl
- pH 7.6

**Isocitrate Dehydrogenase**

As for G6PD but pH 7.8 and use 7.8 mM d, 1 citrate as substrate.

The condition for the preparation and running of the starch gels were:

<table>
<thead>
<tr>
<th>System</th>
<th>Gel Buffer</th>
<th>Bridge Buffer</th>
<th>Volts/cm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGT</td>
<td>0.1M tris:0.01M</td>
<td>As gel</td>
<td>12</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>maleic: 0.001M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EDTA. pH 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADA</td>
<td>0.01M phosphate</td>
<td>As gel</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>pH 6.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>0.01M phosphate</td>
<td>Anode: 0.22M</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>pH 7.3</td>
<td>phospate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 6.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cathode: 0.33M</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phosphate pH 6.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>System</td>
<td>Gel Buffer</td>
<td>Bridge Buffer</td>
<td>Volts/cm</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------</td>
<td>------------------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>ALT</td>
<td>0.1M tris citrate pH 7.5</td>
<td>As gel</td>
<td>10</td>
<td>45</td>
</tr>
<tr>
<td>PGD</td>
<td>As AK</td>
<td>As AK</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td>AcP</td>
<td>0.025M phosphate: 0.015 M citrate pH 5.9</td>
<td>0.245 Phosphate: 0.15 citrate pH 6.45</td>
<td>4.5</td>
<td>46</td>
</tr>
<tr>
<td>G-6 PD</td>
<td>0.05M tris EDTA boric acid pH 8.0</td>
<td>As gel</td>
<td>10</td>
<td>93</td>
</tr>
<tr>
<td>Hp</td>
<td>0.076 M tris: 0.005M citrate pH 8.6</td>
<td>0.3M borate: 0.05 M NaOH pH 8.0</td>
<td>14</td>
<td>93</td>
</tr>
<tr>
<td>AlkP</td>
<td>As Hp</td>
<td>As Hp</td>
<td>13</td>
<td>94</td>
</tr>
<tr>
<td>LNA</td>
<td>As Hp</td>
<td>As Hp</td>
<td>13</td>
<td>95</td>
</tr>
<tr>
<td>Cg</td>
<td>0.01M succinate: 0.0184M tris pH 6.0</td>
<td>M NaOH:0.41M citrate pH 6.0</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Hb</td>
<td>tris; EDTA pH8.6</td>
<td>As gel</td>
<td>14</td>
<td>93</td>
</tr>
<tr>
<td>ICDH</td>
<td>1:10 dilution of bridge buffer</td>
<td>0.1 M tris-citrate pH 7.0</td>
<td>10</td>
<td>92</td>
</tr>
</tbody>
</table>
RESULTS

Serum Polymorphic Systems

Haptoglobin. Table 1 summarises the frequency distribution of the three principal haptoglobin phenotypes in the two test groups with the two sexes analysed separately and combined. Also included in Table 1 are the gene frequencies and estimates and standard errors of the % excess of heterozygotes (58).

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of cases</th>
<th>Haptoglobin phenotypes</th>
<th>Gene frequency</th>
<th>Excess heterozygotes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1 2:1 2:2 Hpl Hp2</td>
<td>Hpl Hp2 estimate</td>
<td>S.E.</td>
</tr>
<tr>
<td>Down's male</td>
<td>141</td>
<td>24 70 47 0.418 0.582</td>
<td>+ 0.98 4.10</td>
<td></td>
</tr>
<tr>
<td>Down's female</td>
<td>113</td>
<td>21 53 39 0.420 0.580</td>
<td>— 1.8 4.58</td>
<td></td>
</tr>
<tr>
<td>Down's combined</td>
<td>254</td>
<td>45 123 86 0.419 0.581</td>
<td>— 0.27 3.0</td>
<td></td>
</tr>
<tr>
<td>Controls male</td>
<td>655</td>
<td>92 353 210a 0.410 0.590</td>
<td>+ 5.4 1.8</td>
<td></td>
</tr>
<tr>
<td>Controls female</td>
<td>198</td>
<td>30 95 73 0.394 0.609</td>
<td>+ 3.8 3.4</td>
<td></td>
</tr>
<tr>
<td>Controls combined</td>
<td>853</td>
<td>122 448 283 0.404 0.596</td>
<td>+ 2.1 1.6</td>
<td></td>
</tr>
</tbody>
</table>

* $\chi^2 = 8.5 (P = 0.01)$.

Neither group when the sexes were combined deviated significantly from the Hardy-Weinberg law, but the male controls did deviate significantly at the $P = 0.01$ level as indicated in Table 1. The gene frequencies of both groups were well within the range of frequencies reported for normal European populations (59 – 62). Ball et al (63) have reported a
Section 2

decrease in the proportion of Down's subjects heterozygous for haptoglobin, and although the data in Table 1 would seem to contradict this the data was subjected to Haldane's log ratio test (64):

\[
\ln \frac{2:1}{1:1 + 2:2} = 0.164 \pm 0.14
\]
i.e. no such deviation occurred in this sample.

The data on the % excess heterozygotes in Table 1 is also in direct contradiction to that of Ball et al (64).

**Group Specific Proteins.** Table 2 compares the group specific protein frequencies of 166 subjects with Down's syndrome and 255 control subjects. The Down's subjects show a considerable excess of Gc 2 : 1 type an excess which by Haldane's log ratio test (64) is significant (0.457 ± 0.201, P = 0.01).

The percentage of heterozygotes in excess of the Hardy-Weinberg law is also significant (+ 4.8%, SE 2.39).
Table 2

Comparison of the Group-Specific Protein Phenotype Frequencies in 166 Subjects with Down’s Syndrome & 255 Control Subjects.

<table>
<thead>
<tr>
<th>Gc Phenotype</th>
<th>Control Subjects</th>
<th>Down’s Syndrome</th>
<th>Down’s Syndrome Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 1</td>
<td>126</td>
<td>63</td>
<td>63.3</td>
</tr>
<tr>
<td>2 - 1</td>
<td>108</td>
<td>91</td>
<td>70.3</td>
</tr>
<tr>
<td>2 - 2</td>
<td>19</td>
<td>12</td>
<td>12.4</td>
</tr>
<tr>
<td>Totals</td>
<td>255</td>
<td>166</td>
<td>166.0</td>
</tr>
</tbody>
</table>

D.S. \( \chi^2 = 0.666 \) control \( \chi^2 = 0.714 \)

The frequency of the Gc allele Gc1 for the combined control group of 0.714 does not differ significantly from the reported frequencies of several European and America (Caucasian) populations (59, 67, 97), and the frequencies of the three phenotypes in the control group do not differ significantly from the Hardy-Weinberg expectation (\( \chi^2 = 0.36 \)).

Table 3 compares the means and standard deviations of the serum levels of the Gc proteins in 119 cases of Down’s syndrome and 55 control subjects. There is a slight reduction in the mean levels of the Down’s group which is just significant at the 5% level. To check if there was any bias to these results due to the three phenotypes migrating at
differing speed (by analogy with the haptoglobins) this data has been sub-divided in Table 3 by phenotypes, and although there appears to be a slight increase due to the \( Gc^1 \) allele, this is not significant at the 5% level.

<table>
<thead>
<tr>
<th></th>
<th>( N )</th>
<th>Mean (mg/100 ml)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down's syndrome</td>
<td>119</td>
<td>29.9</td>
<td>8.96</td>
</tr>
<tr>
<td>Control subjects</td>
<td>55</td>
<td>32.8</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( t = 1.86 ) ( P = 0.05 )</td>
<td></td>
</tr>
<tr>
<td>Down's syndrome</td>
<td>45</td>
<td>30.22</td>
<td>9.57</td>
</tr>
<tr>
<td>(type 1—1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down's syndrome</td>
<td>57</td>
<td>29.7</td>
<td>8.4</td>
</tr>
<tr>
<td>(type 2—1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down's syndrome</td>
<td>10</td>
<td>26.7</td>
<td>3.8</td>
</tr>
<tr>
<td>(type 2—2 ?)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Leucine Naphylamidase Isoenzymes

Fig. 2 shows a photograph of starch gel electrophoresis of a series of sera containing varying amounts of the slower running \( B \)-band. The two outer sera contain no \( B \)-band, while the other four contain from left to right intensities 1—4.

**Fig. 2.** Starch gel electrophoresis of six sera with varying amounts of the leucine naphylamidase \( B \)-band (lower band). From left to right these were rated 0, 1, 2, 3, 4, 0 intensities.
Table 4 compares the frequencies of the intensities on the four point scale of the serum leucine naphylamidase B-bands for Down's and control groups. Also included in this Table are the normal data on 147 Swedish subjects (21).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Low (0+1+2)</th>
<th>High (3+4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down's</td>
<td>160</td>
<td>81</td>
<td>56</td>
<td>16</td>
<td>6</td>
<td>1</td>
<td>153</td>
<td>7</td>
</tr>
<tr>
<td>Controls</td>
<td>83</td>
<td>48</td>
<td>21</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>80</td>
<td>3</td>
</tr>
<tr>
<td>Swedish</td>
<td>147</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>137</td>
<td>10</td>
</tr>
</tbody>
</table>

Compared to the Swedish data there appears to be a decrease in the frequency of the high intensity subjects in both the Down's and control groups, but the rating is highly subjective and this difference probably arises from ratings 2 and 3. Taking the four point rating there is no significant difference between the two test groups ($x^2 = 5.3$) nor is there any difference when classified as low and high intensities ($x^2 = 4.8$).

In one Down's subject (male aged 42 years) an additional band of activity running slower than the B-band was encountered and Fig. 3 shows the starch gel electrophoresis of this subject compared to sera containing no B-band and B-band intensity 3.
Section 2

Fig. 3. Starch gel electrophoretic patterns of serum leucine naphylamidase isoenzymes. From left to right: control serum with B-band intensity 3, control serum no B-band and Down’s subject with an additional slower running band.

To investigate this unusual pattern some 800 sera from normal subjects referred for liver function investigations were investigated and a number of unusual patterns of the isoenzyme were found in addition to the two normal patterns.

A representation of the most common patterns are shown in Fig. 4 below.

Fig. 4. Five types of leucine naphylamidase isoenzyme patterns in subjects showing clinical indications of acute liver dysfunction (jaundice).
The type 3 in Fig. 4 above was found in 23 cases and closely resembles that of the unusual Down's subject. It is probably the same pattern as that reported elsewhere (90) in six cases of acute infective hepatitis. Subsequent testing of this serum for the hepatitis associated antigen (see Section 4) confirmed this as a diagnosis. In all the cases of type 3 pattern this additional slower band is always associated with the presence of high intensity B-band and preliminary studies (23) have suggested that they are not of pancreatic origin as both are inhibited with methionine.

Serum Alkaline Phosphatase. Table 5 summarises the frequency distribution of the two principal alkaline phosphatase types in 241 subjects with Down's syndrome with the Pp2 types also segregated according to their ABO blood group.

<table>
<thead>
<tr>
<th>Blood group</th>
<th>Type Pp1 found</th>
<th>Type Pp1 expected</th>
<th>Type Pp2 found</th>
<th>Type Pp2 expected</th>
<th>Percentage of type Pp2 this investigation</th>
<th>Percentage of type Pp2 Beckman (1964)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>87</td>
<td>90.3</td>
<td>23</td>
<td>19.6</td>
<td>21.0</td>
<td>30.4</td>
</tr>
<tr>
<td>A</td>
<td>82</td>
<td>73.1</td>
<td>7</td>
<td>15.9</td>
<td>7.7</td>
<td>2.3</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>30.4</td>
<td>12</td>
<td>6.6</td>
<td>32.4</td>
<td>30.5</td>
</tr>
<tr>
<td>AB</td>
<td>4</td>
<td>4.1</td>
<td>1</td>
<td>1</td>
<td>20.0</td>
<td>15.2</td>
</tr>
<tr>
<td>Total</td>
<td>188</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The overall frequency of 17.8% for the Pp2 type does not differ from that reported in normal subjects (Beckman (65)) ($\chi^2 = 1.96$). Segregation by ABO blood groups shows a decrease
of type A in Pp2 subjects over type B with type AB falling intermediate between them. This general trend has been well established for normal populations and comparison of the test data with that of Beckman (65) in Table 5 shows the similarity between normals and Down's subjects in this respect.

\( \beta_2 \)-Glycoprotein. Fig. 5 compares the \( \beta_2 \)-glycoprotein distributions of 91 control subjects and 98 Down's subjects. Comparison of these distributions with those of normal subjects shown elsewhere in this section (Fig.1) should be made. In Fig. 5 the vertical bars indicate the phenotype mean and the horizontal bars are \( \pm 1 \text{SD} \) from this mean. Since liver disfunction is known to affect the levels of this protein data on 45 jaundiced normal subjects are included in Fig. 5.
From the t-tests shown in Table 6 there were no significant differences between means either for the two test groups combined or between the phenotypes of the two groups. The frequency of Bg N gene was found to be: for the Down's subjects 0.944 (SE 0.016) and for the control group 0.939 (0.018) a difference which is not significant. Neither groups deviated from the Hardy-Weinberg law ($x^2 = 0.346$ and 0.376 for Down's and control groups), and the gene frequencies of both groups are similar to that reported for normal German (0.953) (56) and English populations (0.941) (66).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Down's</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean</td>
</tr>
<tr>
<td>N : D</td>
<td>11</td>
<td>11.34</td>
</tr>
<tr>
<td>N : N</td>
<td>87</td>
<td>18.96</td>
</tr>
<tr>
<td>D : D</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Combined</td>
<td>98</td>
<td>18.10</td>
</tr>
</tbody>
</table>

**Serum Cholinesterase C$_5$**

Serum cholinesterase C$_5$ typing was carried out on 100 subjects with Down's syndrome (19 of whom were positive) and 95 control subjects (17 of whom were positive). This frequency difference was not significant.
Red Cell Polymorphic Systems.

Haemoglobin, Isocitrate Dehydrogenase and Glucose-6PD. In the presentation below these three systems will not be included. In 150 subjects examined for the ICDH system all were found to be phenotypically 1:1 as would be expected for a Caucasian population. No examples of the rare haemoglobin types were encountered, and one rare variant of the A type of G-6PD was found, and this will be described elsewhere.

The phenotype frequencies of the remaining six red cell polymorphic systems are given in Table 7 below.

To investigate whether or not there is either sex or age effects on the distribution of these phenotype systems the Down's sample was sub-divided by sex and into three age categories, 0 - 18 years, 19 - 28 years, 29 - 63 years. The data from Table 7 so subdivided is presented in Table 8 below.
Section 2

Table 7. Phenotype frequencies of six red cell polymorphic systems in Down's subjects and in control subjects.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Phenotype</th>
<th>Down's syndrome Found</th>
<th>Expected</th>
<th>Controls Found</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGT</td>
<td>1:1</td>
<td>70</td>
<td>71.4</td>
<td>114</td>
<td>118.2</td>
</tr>
<tr>
<td>(males)</td>
<td>2:1</td>
<td>60</td>
<td>57.15</td>
<td>82</td>
<td>73.4</td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>10</td>
<td>11.45</td>
<td>7</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>140</td>
<td></td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>PGT</td>
<td>1:1</td>
<td>72</td>
<td>71</td>
<td>124</td>
<td>125.8</td>
</tr>
<tr>
<td>(females)</td>
<td>2:1</td>
<td>36</td>
<td>37.9</td>
<td>77</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>6</td>
<td>6.1</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>114</td>
<td></td>
<td>203</td>
<td></td>
</tr>
<tr>
<td>ADA</td>
<td>1:1</td>
<td>221</td>
<td>218.2</td>
<td>374</td>
<td>370.9</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>30</td>
<td>33.5</td>
<td>40</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>3</td>
<td>1.29</td>
<td>4</td>
<td>1.40</td>
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The expected frequencies are based on the system obeying the Hardy-Weinberg law.
Table 8. Phenotype frequencies in Table 7 sub-divided by age and sex.

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<th>System</th>
<th>Phenotype</th>
<th>0-18 Years</th>
<th>19 - 28</th>
<th>29 - 63</th>
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<td>Female</td>
<td>Male</td>
<td>Female</td>
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<td>0</td>
<td>0</td>
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</tr>
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<td>32</td>
<td>27</td>
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<td>27</td>
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<td>total</td>
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<td>26</td>
</tr>
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<td>29</td>
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<td>15</td>
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</tr>
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<td>12</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
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<td>total</td>
<td>12</td>
<td>14</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>
Section 2

With the exception of the PGT1 system no significant effects of either age or sex could be detected. Comparison of the combined males x combined females for the Down's subjects revealed a significant increase in heterozygote frequency ($x^2 = 10.5, P = 0.01$) and it can be shown that this is entirely due to the younger age group (0 - 18 years) ($x^2 = 16.7, P = 0.001$) with the other two age groups showing no difference ($x^2 = 2.14$ and $3.24$).

In Table 9 below the distribution data from Table 7 has been subjected to further analysis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Allele</th>
<th>Gene frequency</th>
<th>s.e.</th>
<th>Estimate</th>
<th>s.e.</th>
<th>Deviation from H-W freq</th>
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<tr>
<td>PGT1</td>
<td>PGT1</td>
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<td>0.0227</td>
<td>2.02</td>
<td>1.7</td>
<td>0.35</td>
</tr>
<tr>
<td>(male)</td>
<td>PGT1</td>
<td>0.205</td>
<td>0.0227</td>
<td>-1.7</td>
<td>1.55</td>
<td>0.205</td>
</tr>
<tr>
<td>PGT1</td>
<td>PGT1</td>
<td>0.211</td>
<td>0.021</td>
<td>+0.8</td>
<td>2.97</td>
<td>1.03</td>
</tr>
<tr>
<td>(female)</td>
<td>PGT1</td>
<td>0.51</td>
<td>0.021</td>
<td>+0.8</td>
<td>2.97</td>
<td>1.03</td>
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<td>0.44</td>
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<td>0.011</td>
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<td>A</td>
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</table>

Table 9. Analysis of the frequency distributions of six red cell polymorphic systems.
Comparison of the gene frequencies of the two groups failed to reveal any differences, and with the exception of the ALT system there were no differences between the Down's group and the reported gene frequencies for normal European populations (67 - 69). In a recent review, the gene frequency of $ALT^1$ allele was shown to vary in European populations from 0.48 to 0.54 (70) which is in agreement with American data (70), and this is somewhat higher than either the Down's or control groups in this investigation.

In addition to the failure to detect any differences between the gene frequencies in Table 9 no differences by the Haldane log ratio test could be detected. A slight reduction of the proportion of heterozygotes from that expected by the Hardy-Weinberg law was found in the AK system and in the PGD systems of the Down's subjects.

Tables 10 and 11 summarise the possible first order interactions among the phenotypes of the six red cell systems and the Gc and Hp serum polymorphisms. These were carried out using the G-statistic as outlined in the Introductionary section (72). Significant associations could be detected between the PGT and AcP genes in the Down's group and between
the Hp/AcP and Hp/ADA in the control groups. This investigation failed to confirm the report (99) of an association of AcP allele A with age in the Down's subjects, but did confirm a report (73) of a significant association between AcP and Hp in the control subjects whereas no such association existed in the Down's group.

Table 10

G-tests for the degree of association between phenotypes of the Down's subjects.

<table>
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<tr>
<th></th>
<th>ADA</th>
<th>ACP</th>
<th>AK</th>
<th>PGD</th>
<th>ALT</th>
<th>Hp</th>
<th>Gc</th>
<th>ABO</th>
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<tbody>
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<td>4.00</td>
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<td>8.60</td>
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* * P = 0.01.
Table 11

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<th>ALT</th>
<th>PGD</th>
<th>Hb</th>
<th>Gc</th>
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* P = 0.05
** P = 0.001

ABO Blood Group System

Table 12 below compares the blood group distribution of the 256 Down's subjects in this investigation with a number of surveys previously reported.

Table 12

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<tr>
<th>Population</th>
<th>Total</th>
<th>A</th>
<th>B</th>
<th>O</th>
<th>A &gt; O</th>
<th>References</th>
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<td>Austria</td>
<td>44</td>
<td>21</td>
<td>7</td>
<td>1</td>
<td>15</td>
<td>Yes Oriel (1927)</td>
</tr>
<tr>
<td>Germany</td>
<td>26</td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>11</td>
<td>Yes Mannitz (1932)</td>
</tr>
<tr>
<td>England</td>
<td>166</td>
<td>83</td>
<td>14</td>
<td>3</td>
<td>65</td>
<td>Yes Penrose (1932)</td>
</tr>
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<td>U.S.A.</td>
<td>125</td>
<td>48</td>
<td>12</td>
<td>5</td>
<td>60</td>
<td>No Benda &amp; Bixby (1939)</td>
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<td>3</td>
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<td>Yes Engler (1940)</td>
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<tr>
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<td>63</td>
<td>9</td>
<td>5</td>
<td>61</td>
<td>No Lang Brown et al. (1953)</td>
</tr>
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<td>4</td>
<td>24</td>
<td>Yes Hauckel (1954)</td>
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<td>94</td>
<td>25</td>
<td>323</td>
<td>Yes Shaw &amp; Gershowitz (1952)</td>
</tr>
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<td>34</td>
<td>15</td>
<td>72</td>
<td>No Kaplan et al. (1964)</td>
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<td>32</td>
<td>7</td>
<td>113</td>
<td>No Present Investigation</td>
</tr>
</tbody>
</table>
Section 2

Even casual inspection of the data in Table 12 shows the divergent findings of both Penrose (5) and Lang Brown (2) with that of this investigation, although all three samples were drawn from the same geographical area. The frequency of type B in the present sample is much greater than that found in the two surveys quoted and is similar to the data of Shaw and Gershovitz (8). Using the method of least likelihood (74) the frequency of the A gene was found to be 0.305, the B gene 0.08 and the O gene 0.615.

Rare Variants Detected

Several rare variants among the control sample were detected during the course of this investigation, these included several examples of abnormal haemoglobin, one example of the AK phenotype 3 : 1, but further investigation of these were outside the terms of reference for this thesis and will not be reported upon.

In the Down's group, reference has been made above to an unusual pattern of leucine naphylamidase isoenzymes which was possibly due to liver damage. Several examples of reduced haptoglobin levels were encountered which could be interpreted as examples of the silent Hp^0 gene, but as has been shown
elsewhere (75) these are more likely to be cases of hypohapto-globinaemia secondary to liver disorders than heterozygote for the silent gene.

One example of PGT locus 2 type 3 : 1 was detected in a male subject with Down's syndrome, and subsequent investigation of the family failed to detect this variant in either the mother or the presumptive father and presumptive sibs. Some doubt on the parenthood of the propositus was cast and cooperation of the family for full blood grouping to establish paternity was refused. Fig. 6 below shows the photograph of the starch gel for the PGT$_2$ locus, haemolysate a, b and d are all PGT$_2$ 1 : 1 and haemolysate c is the 3 : 1 variant.
Fig. 7 is a starch gel electrophoresis of the G-6PD system, haemolysates a, b and d are the common variant (type B) whereas sample c constantly showed an additional band of activity running faster than the B variant and is probably the A variant common in coloured populations. Investigation of the family proved abortive as they were reported to have 'gone abroad' and as some doubt is cast on the Caucasian origin of this subject all data on this person was removed prior to analysis of the Down's group.

In one Down's subject (female aged 50 years) an unusual variant of the PGD system was detected. Two bands of activity
were detected which differed in mobility from the position of the AB homozygous form. Fig. 8 below compares electrophoretic patterns of this subject (haemolysate b) with the common phenotype PGD A : A (samples a, c and d) and the rarer homozygous PGD A : B.

Fig. 8

![Electrophoretic pattern](image)

Fig. 8b

![Additional electrophoretic pattern](image)
Fig. 8b above is a diagrammatic representation of the PGD patterns in Fig. 8. Sample e is the unusual variant, sample b is PGD type A : B and the other samples are the common type A.

Examination of the literature has failed to produce another example of this variant. The parents of this subject are now deceased, but one normal sib and several relatives (cousins, aunts, etc.) all failed to reveal this variant and the mode of inheritance, whether an inherited variation or a sporadic mutation remains conjecture.
DISCUSSION

The finding of a normal phenotype distribution in the haptoglobin system confirms the work of earlier investigators (10, 11), but fails to confirm the observation of Brackenridge et al (73) of an excess of type 1 : 1 in male Down's when compared with female Down's subjects, within their sample the males heterozygotes exceeding the control sample by 10.5%. One possible explanation for this divergence in findings was suggested by the observation of an apparent maternal age effect on the proportion of heterozygotes for haptoglobin in male Down's subjects but not in female Down's subjects (63).

This aspect will be investigated in depth in Section 4.

The data on the leucine aminopeptidase patterns is more complex than the simple genetic control as shown by most of the systems investigated. The presence of this B-band is only partially under genetic control, and is affected by external factors such as the administration of oral contraceptives (22), and drugs such as the phenothiazine and barbiturate derivatives (23, 65). The hypothesis that the B-band is a
Section 2

sensitive indicator of altered liver function (22) is hard to reconcile with the finding of normal B-band intensities in the Down's subjects in this investigation, as Down's subjects are known to be more susceptible to liver damage resulting from increased susceptibility to infections such as the long-incubation period form of hepatitis (discussed further in Section 4).

The significant shift of the phenotype frequencies of the Gc proteins in the Down's group is suggestive, at first sight, of the location of this system on the trisomic 21st chromosome. However, if one were to propose a simple dosage effect one could anticipate a 50% increase in the quantitative levels of this protein in the serum which is manifestly not the case. Further evidence against locating the Gc proteins on the trisomic chromosome can be evinced from the data on the albumin levels in Down's syndrome. In Section 1 the literature on the levels of serum proteins was surveyed, and perhaps the most consistent finding was a reduced albumin level, a finding true of the present sample. The locus for the Gc proteins has been shown to be closely linked with that of albumin (80) which should be elevated if the two loci were located on chromosome 21. To propose that the Gc locus is on
Section 2

the 21st chromosome would require some unjustified hypothesis that the appearance of the phenotypes in the blood originating in the liver (81) must be under some unusual form of control in the Down's subjects. There are three possible mechanisms which may account for the appearance of an excess of the heterozygotes in the blood without recourse to suggesting the location of the locus on the trisomic chromosome. The first possibility is that the frequency of the heterozygotes are in some way affected by the maternal age of the subjects (by analogy with the haptoglobin system as will be shown in Section 3), this possible mechanism will be investigated in Section 3. The second arises from the observation of possible association between the Gc 1 : 1 phenotype and the presence of long-incubation hepatitis virus in Down's syndrome, this possibility will be considered in Section 4. The third possibility follows the observation on family data by Reinskou (83) that the Gc genotype may influence the survival of the foetus and in a condition such as Down's syndrome where pre-natal and peri-natal death rates are high, even a marginal advantage could cause selection by mortality. This possibility will be investigated in Section 5.

The genetic mechanisms for the appearance in the blood of the
intestinal form of alkaline phosphatase (Pp2) requires not only the interaction of the ABO locus and the secretor status, but also the presence of other unspecified 'genetic factors' (28) and the correct response to the effects of diet (29). The evidence of this investigation suggests that although there may be slight changes in one or more of the individual mechanisms listed above the overall effect is neutral.

The failure to detect any differences in the frequencies of the red-cell systems investigated confirms the findings of several surveys published after the start of this investigation (10, 77) but it should be noted that in a more recent communication (73) one of these authors have shown a decrease in the proportion of heterozygotes for the PGT system in female Down's subjects compared with either male Down's or control females. The sample of Down's subjects in this survey also showed this slight decrease (31.6% against 42.8%).

The increase in proportion of PGT type 1 : 1 in males under 18 years probably reflects age trends shown by other surveys (73, 71), and the mechanism for this is not clear but a recent report has been made of an excess of heterozygotes
Section 2

found among children, but not their parents, in Danish, German and English families and that the PGT locus is subject to selection (77).

The finding elsewhere of a significant decrease in females with Down's syndrome of red cell acid phosphatase type AB and an age trend associated with a decrease in type A with increasing age (73) could not be confirmed. However, if the age trend is true, then a fortuitous selection in ages of the Down's subjects may counteract the former effect.

Probably the most interesting feature of this section was the observation of an apparent association between the acid phosphatase and haptoglobin in the control group but not in the Down's group, an observation confirmed elsewhere (84). It has been suggested (84) that the association between these two systems may be an example of conditional heterozygote advantage with heterozygosity in one system being favoured by heterozygosity in the other. This, however, is pure speculation, but it is known that at least the two loci are not closely linked (85, 86).

Considerable interest has been focused on the possibility
that the ABO locus may be situated on the G chromosome (87). Shaw and Gershowitz (8) showed a deficit of group 0 and an excess of Group A and B, a situation which could arise from the contribution of two alleles from one parent. The confirmation of this by observing an AB child from 0 and AB parents is still not forthcoming and it is still necessary to rely on changes in phenotype frequencies to strengthen or weaken the case. Normal ABO frequencies in England (88) from a study of more than 190,000 individuals analysed by the technique of Fisher and Taylor (89) showed a frequency of 0 gene 0.683, B = 0.06 and A = 0.257. Adopting the same technique the frequencies in this investigation for the Down's group yielded: 0 = 0.653, A = 0.257 and B = 0.087, i.e. an excess of group B at the expense of group 0 (significant at P = 0.05). Of the nine surveys quoted above, six have shown an excess of group A over 0, and two the reverse. It should be noted however that Down's subjects have been shown to be more compatible with their mothers than fathers and selection against ABO incompatible offspring is highly likely (79). If this is so, further family studies may yet provide valuable information on the causation of non-disjunction and its possible relationship to specific phenotypes.
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SECTION 3

THE EFFECT OF PARENTAL AGE ON SOME

PHENOTYPE FREQUENCIES IN DOWN'S SYNDROME
Section 3

INTRODUCTION

The association between Down’s syndrome and parental age has been known for some time, with Fraser and Mitchel (1876) (1) being credited with first producing evidence of late maternal age effect, and subsequently Penrose (2) and Jenkins in the same year (1932) (3) showing that the age of the father was irrelevant. Two years later Penrose was able to show that the maternal age effect was independent of parity (4).

Recently Richards (5) has been able to point out "statistical enquiries have contributed greatly to our understanding of mongolism during its one hundred years history, but it is doubtful if much more information can be squeezed out of the available data by its use. What we require is more detailed information about the biology of the mother, particularly about oogenasis and the surrounding physiological and pathological events". Burch (6), from an analysis of log-log graphs of the relative frequency of births of Down’s subjects in control populations against maternal age for English and Swedish populations has deduced that at least ten random events initiate the pathogenic processes responsible for most offspring with Down’s syndrome born to
mothers of late maternal age.

In their analysis of polymorphic frequencies Ball et al (7) observed a positive linear regression of the proportion of male Down's subjects heterozygous for the haptoglobin system on maternal age, and more recently the same laboratories (8) reported similar positive regressions for the PGT system. It may be possible that one or more of the random events as outlined by Burch (6) may involve either the phenotype of the offspring or possibly maternal/foetal incompatibility.

In this section, the possible relationship between parental age and five polymorphic systems will be considered, and an analysis to assess the possible foetal/maternal incompatibility will be attempted.

Family Data

The age of the father and mother of all the subjects investigated in this section was obtained to the nearest year from the required form completed on admission of the subject to hospital. In those cases where these were either incomplete or equivocable, additional information was requested from next of kin. From similar sources the number, sex and birth
order of all sibs (including still births and miscarriages) were obtained. Of the original sample of 256 Down's subjects, reliable information was obtained on 221 cases. During the course of this investigation, blood was obtained from the parents of 94 subjects with Down's syndrome.

**Selection of Parental Age Groups**

In an attempt to minimise bias, cumulative frequency curves for both paternal and maternal ages was drawn up for both the Down's and control groups. The parental age groups were then selected in terms of the tenth percentiles rounded off to the nearest whole year providing ten approximately equal groups.

**Regression of Heterozygote Frequency on Parental Age.**

In order to make the analysis comparable with that reported elsewhere (7) the proportion of heterozygotes to homozygotes was transformed into the arc-sine transformation as outlined in the introductory section. The subsequent equations of regression, the 95% confidence limits and the deviation of the coefficient of regression from zero were carried out using standard procedures (9).
Section 3

Estimation of Parity Effect.

The effect of parity on the frequency of the heterozygotes for haptoglobin in Down's syndrome was determined using the method described by Penrose (4). Unfortunately, without phenotyping the haptoglobins of all the sibs (some 900) a complete analysis of the data could not be performed. As these 900 sibs were distributed throughout the whole United Kingdom such a survey was outside the scope of this thesis.
RESULTS

Table 1 shows the distribution of the sibships in the sub-sample of 221 Down's subjects. Using the method of Penrose (4), a birth order effect could be detected ($x^2 = 64.8$, $P = 0.01$) but as Penrose has suggested (9) this birth order effect is probably secondary to the late maternal age effect.

Table 1

<table>
<thead>
<tr>
<th>Position in sibship</th>
<th>Number of sibship</th>
<th>Found Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23 22 11 6 1</td>
<td>63 82.4</td>
</tr>
<tr>
<td>2</td>
<td>28 8 3 4 1</td>
<td>44 59.4</td>
</tr>
<tr>
<td>3</td>
<td>33 5</td>
<td>38 44.4</td>
</tr>
<tr>
<td>4</td>
<td>15 2</td>
<td>17 17.1</td>
</tr>
<tr>
<td>5</td>
<td>11 1</td>
<td>12 9.85</td>
</tr>
<tr>
<td>6</td>
<td>11 1</td>
<td>12 6.6</td>
</tr>
<tr>
<td>7</td>
<td>8 1</td>
<td>10 4.15</td>
</tr>
<tr>
<td>8</td>
<td>7 2</td>
<td>9 3.03</td>
</tr>
<tr>
<td>9</td>
<td>5 1</td>
<td>6 2.03</td>
</tr>
<tr>
<td>10</td>
<td>3 2</td>
<td>5 1.13</td>
</tr>
<tr>
<td>11</td>
<td>3 1</td>
<td>0 0.5</td>
</tr>
<tr>
<td>12</td>
<td>3 1</td>
<td>0 0.32</td>
</tr>
<tr>
<td>13</td>
<td>0 0.6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0 0.06</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0 0.06</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1 0.06</td>
<td></td>
</tr>
</tbody>
</table>

23 52 29 16 15 8 8 8 3 3 3 2 0 0 1

Tables 2 and 3 below show the data in Table 1 sub-divided into sibships in which the Down's subject is homozygous or heterozygous for haptoglobin.
A birth order effect similar to the combined Down's group could be detected for both the samples homozygous and heterozygous for haptoglobin (Tables 2 and 3) ($\chi^2 = 23.9$ and 28.5 respectively, $P = 0.001$).

The correlation between parental age and frequency of Down's
syndrome was estimated and found to be, for paternal age $r = 0.22$ (SE 0.17) and for maternal age $r = 0.49$ (SE 0.09) and the intercorrelation between maternal and paternal age to be $r = 0.73$ (SE 0.09). Partialling out the effect of paternal on maternal age, no significant effect of paternal age on the frequency of Down's syndrome could be shown (partial $r = 0.12$).

Fig. 1 below shows the distribution of the ages of the mothers and fathers of the Down's subjects.

---

**Parental Ages and Haptoglobin Frequencies**

Figs. 2 and 3 below show the effect of parental ages on the haptoglobin frequencies (as the arc-sine transform) of the
males and females with Down's syndrome treated separately (Fig. 2) and combined (Fig. 3). Both the male and female subjects with Down's syndrome show similar distributions against both paternal and maternal ages, i.e. a bimodal distribution with the modal point at about 35 years for the maternal age and 40 years for paternal age. Fig. 3 also includes the data on 120 control subjects similarly treated (broken curve), and no bimodality can be detected.

A bimodality such as has been shown to occur above causes considerable analytical problems, for example correlations
require linearity. The two modes can be compared with test such as the Haldane log ratio test with some expectation of reliability (10) and the data for such a test is shown in Table 4 below.

<table>
<thead>
<tr>
<th>Mat. age group (years)</th>
<th>Number of D.S. subjects heterozygous for Hp.</th>
<th>Number of D.S. subjects homozygous for Hp.</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>17—30</td>
<td>33</td>
<td>24</td>
<td>57</td>
</tr>
<tr>
<td>31—46</td>
<td>74</td>
<td>89</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>107</td>
<td>113</td>
<td>220</td>
</tr>
</tbody>
</table>

Table 4. Maternal age

Numbers of Down's syndrome subjects homozygous and heterozygous for serum haemoglobin for two maternal age groups 17—30 years and 31—46 years.

From Table 4 the estimate of the log ratio test was found to be 0.49 (SE 0.077) i.e. the difference between the two modes was significant at $P = 0.01$. With less reliability, each mode could be considered as a homogeneous group with a linear relationship between the arc-sine transform and parental age, for example in the combined Down's group, for maternal age mode 30+, the regression equation can be shown to be:

$$\text{arc-sine transform} = 1.39 \text{ age} - 12.3$$

and the correlation between maternal age (30+) and the transform to be $r = 0.93$ ($P = 0.001$).
**Parental Age and Other Phenotypes**

Figs. 4 - 7 show the regressions of the arc sine transforms of the serum Gc protein, red cell acid phosphatases, red cell PGT and ALT systems, on age.

---

**Fig. 4.** Relationship between the arc-sine transformations of the ratio of heterozygous/homozygous frequencies of the serum Gc system and parental age. Solid lines represent the equation of the regression and the broken lines the 95% confidence limits.

**Fig. 5.** Relationship between the arc-sine transformations of the ratio of the heterozygous/homozygous frequencies of the red cell acid phosphatase system and parental age. The solid line represents the equation of the regression and the broken lines the 95% confidence limits.

**Fig. 6.** Relationship between the arc-sine transformation of the ratio of the heterozygous/homozygous frequencies of the red cell phosphoglucomutase system and parental age. The solid line represents the equation of regression and the broken lines the 95% confidence limits.

**Fig. 7.** Relationship between the arc-sine transformation of the ratio of the heterozygous/homozygous frequencies and parental age of the red cell glutamic-pyruvic transaminase system and parental age. The solid line represents the equation of the regression and the broken lines the 95% confidence limits.
Section 3

In Figs. 4 - 7 the unbroken lines indicate the equation of the regression and the broken lines the 95% confidence limits of this equation. The data from which these equations are derived are shown in Table 5 below, and as can be seen from the t-test for significance of the regression coefficients in this Table in none of the four systems is there a significant deviation from zero. This finding is further supported by the lack of correlation between the arc-sine transforms and maternal age. For comparison the haptoglobin for maternal age 30+ was shown to be $r = 0.93$, for the other systems the values of $r$ were $0.07$ (AcP), $0.42$ (Gc), $0.16$ (PGT) and $0.31$ (ALT), none of which are significant at $P = 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>AcP</th>
<th>Bcm</th>
<th>Gc</th>
<th>Gff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mat.</td>
<td>Pat.</td>
<td>Mat.</td>
<td>Pat.</td>
</tr>
<tr>
<td>Regression coefficient (b)</td>
<td>$-0.096$</td>
<td>$-0.003$</td>
<td>$-0.202$</td>
<td>$-0.08$</td>
</tr>
<tr>
<td>$y$ — intercept</td>
<td>50.3</td>
<td>47.4</td>
<td>46.8</td>
<td>33.6</td>
</tr>
<tr>
<td>Significance of b (t-test df = 8)</td>
<td>$0.247$</td>
<td>$0.27$</td>
<td>$0.39$</td>
<td>$0.36$</td>
</tr>
<tr>
<td>Mean of arc sine transform ($\bar{y}$)</td>
<td>46.97</td>
<td>47.28</td>
<td>39.8</td>
<td>34.6</td>
</tr>
<tr>
<td>S.E. of $\bar{y}$</td>
<td>1.87</td>
<td>2.1</td>
<td>2.05</td>
<td>2.83</td>
</tr>
</tbody>
</table>

Summary of the data of the regression of the arc-sine transformation of the heterozygous/homozygous frequencies of one serum (Gc) and three red cell polymorphic systems.

Family Phenotype Data

Table 6 shows the phenotype distributions of 94 parents of Down's subjects for the red cell acid phosphatase system together with the expected frequencies based on the normal data of Hopkinson & Harris (11).
### Table 6

<table>
<thead>
<tr>
<th>Mating Mother</th>
<th>Mating Father</th>
<th>Frequency Found</th>
<th>Frequency Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A:A x A:A</td>
<td></td>
<td>2</td>
<td>1.83</td>
</tr>
<tr>
<td>A:A x A:B</td>
<td></td>
<td>6</td>
<td>5.98</td>
</tr>
<tr>
<td>A:A x B:B</td>
<td></td>
<td>3</td>
<td>4.9</td>
</tr>
<tr>
<td>A:B x A:A</td>
<td></td>
<td>7</td>
<td>5.98</td>
</tr>
<tr>
<td>A:B x A:B</td>
<td></td>
<td>13</td>
<td>19.63</td>
</tr>
<tr>
<td>A:B x B:B</td>
<td></td>
<td>19</td>
<td>16.09</td>
</tr>
<tr>
<td>B:B x A:A</td>
<td></td>
<td>5</td>
<td>4.9</td>
</tr>
<tr>
<td>B:B x A:B</td>
<td></td>
<td>19</td>
<td>16.09</td>
</tr>
<tr>
<td>B:B x B:B</td>
<td></td>
<td>16</td>
<td>13.18</td>
</tr>
<tr>
<td>A:B x A:C</td>
<td></td>
<td>0</td>
<td>2.72</td>
</tr>
<tr>
<td>A:C x A:B</td>
<td></td>
<td>0</td>
<td>2.72</td>
</tr>
</tbody>
</table>

The chi square test failed to reveal any significant variation in the mating frequencies of the parents of Down's subjects as might have been expected if severe maternal/foetal incompatibility was the major cause of the syndrome ($x^2 = 10.33$).

In Table 7 below the mating frequencies of the Gc, PGT and Hp systems are shown.
### Table 7

<table>
<thead>
<tr>
<th>Parenting</th>
<th>Gc Found</th>
<th>Expected</th>
<th>PGT Found</th>
<th>Expected</th>
<th>Hp Found</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1 x 1:1</td>
<td>31</td>
<td>24.4</td>
<td>34</td>
<td>32.9</td>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>1:1 x 2:1</td>
<td>16</td>
<td>19.6</td>
<td>17</td>
<td>19.5</td>
<td>7</td>
<td>5.46</td>
</tr>
<tr>
<td>1:1 x 2:2</td>
<td>4</td>
<td>3.9</td>
<td>2</td>
<td>2.99</td>
<td>4</td>
<td>7.7</td>
</tr>
<tr>
<td>2:1 x 1:1</td>
<td>17</td>
<td>19.6</td>
<td>22</td>
<td>19.5</td>
<td>15</td>
<td>11.5</td>
</tr>
<tr>
<td>2:1 x 2:1</td>
<td>12</td>
<td>15.7</td>
<td>15</td>
<td>12.0</td>
<td>7</td>
<td>5.5</td>
</tr>
<tr>
<td>2:1 x 2:2</td>
<td>2</td>
<td>3.1</td>
<td>1</td>
<td>1.84</td>
<td>17</td>
<td>16.0</td>
</tr>
<tr>
<td>2:2 x 1:1</td>
<td>6</td>
<td>3.9</td>
<td>2</td>
<td>2.99</td>
<td>5</td>
<td>7.67</td>
</tr>
<tr>
<td>2:2 x 2:1</td>
<td>4</td>
<td>3.14</td>
<td>1</td>
<td>1.85</td>
<td>16</td>
<td>15.9</td>
</tr>
<tr>
<td>2:2 x 2:2</td>
<td>1</td>
<td>0.63</td>
<td>0</td>
<td>0.28</td>
<td>21</td>
<td>21.9</td>
</tr>
</tbody>
</table>

Chi-square: 5.8  3.13  4.75

As indicated by the chi-squares in Table 7 above in none of these systems is there any evidence of deviations from the normal expected frequencies.
DISCUSSION

The distribution of age of mothers at the birth of a Down's child is now very familiar, having the peculiarity of being bimodal or at least bitangential, which has been interpreted as being the result of two classes of affected subjects, the first not subject to maternal influences forming that part of the curve which resembles that for the general population and those subject to maternal influence. These two classes have been termed class A and class B respectively (14) with class A varying from one third to one half of most samples examined. This distinction of class does not imply a difference between the two classes of Down's subjects themselves, but rather differences in possible causation of the non-disjunction giving rise to the syndrome. In fact both classes of Down's subjects when investigated are found to be chromosomally and clinically indistinguishable or occasionally a small proportion of class A being chromosomally different (usually in the form of translocations), class A subjects are smaller in number than class B and are not homogeneous for cause. An important aetiological distinction to make is that between subjects whose condition arise primarily from meiotic error in the mother and those in whose
case a chromosomal anomaly was already present in the somatic cells of the mother. It would appear at first sight that the selection of Down's subjects limited to those trisomic for chromosome 21 would have eliminated those cases in which a balanced translocation occurred in the mother, but as will be seen later this is not necessarily so. It has been suggested (15) that there is a general tendency to meiotic errors in some families, if so it would be heritable and therefore show itself by a familial tendency and such Down's subjects would be class A maternal age independent. In a collection of reported data (15) on Down's subjects with similarly affected relatives it has been shown that when the relative was related on the mother's side the mean maternal age of both affected subjects (although raised above the normal) failed to reach that of the condition in general, but there was no such weakening of the maternal age effect when they were related on the father's side. These observations suggest a sex-influenced or sex-limited genetical tendency towards the occurrence of meiotic errors. A survey of 130 Down's subjects (5) showed an incidence of D/G translocations and three families with one subject as a D/G translocation and another with trisomy. It thus appears that class A subjects may be caused by translocation carriers or by sporadic translocations.
or by some heritable tendency. A further cause may be maternal translocation which has been shown to occur but is difficult to detect chromosomally. Penrose (16) judging by the results of dermatoglyphs has estimated that about 10% of all Down's subjects might have mothers who are themselves mosaics as indicated by a raised atd angle. Such subjects born to mosaic mothers are class A so the mean maternal age should be the same as for the general population. However the evidence available (17) is suggestive of most mosaic mothers starting as regular trisomics which in early embryogenesis underwent a meiotic error, leading to mosaicism, i.e. the mothers themselves started life as class B so the mean grandmaternal age is raised. Burch (6) has presented log-log graphs of the relative frequencies of Down's births in three populations, all of which showed the same general features, age dependence is slight from 15 to 25 years but after 30 years the relative frequency (P) of Down's births rises with a high power of age. Each of the three sets of data presented can be described by the sum of two mathematical functions, the first attaining a plateau at \( t = 20 \) years, the second obeys the relationship \( P \propto t^{10} \) up to 40 years, beyond which the steepness of age dependence declines slightly. \( P \) can be regarded as proportional to the age specific prevalence of women who are likely to produce an affected child.
and probably the two mathematical functions relating $P_t$ to $t$ represent particular cases of a general law (20, 21). The same author has shown that the age prevalence of many diseases ($P_t$) and ageing condition of the so-called autoimmune (autoaggressive) disorders can be described by the general stochastic equation:

$$P_t = S (1 - (\exp^{-kt^r})^n) \ldots \ldots \ldots \ldots (1)$$

where $P_t$ is the age specific and sex specific prevalence of initiated disease at time $t$, $S$ is equal to or proportional to the fraction of the general population predisposed to the disease at birth, $r$ is a positive integer from 1 to 5 (20).

From these equations Burch (6) offered a unified interpretation of the effect of maternal age on the frequency of Down's syndrome. Within the population there are two subgroups $S_1$ and $S_2$ (Class A and B of Penrose) which are predisposed to produce offspring with the syndrome. In the younger mothers the penetrance of the condition is effectively complete by 20 years although the number of random events required to initiate the condition cannot be determined by present data. Probably ten random events are
required to initiate the pathogenic process responsible for most of the offspring born to the older sub-group of mothers (class B), i.e. the value of \( n \) (or more likely \( nr \)) in equation (1) above is 10. An average interval (latent period) of about 2.5 years elapses between the last initiating event and the first expression of the disorder in the form of non-disjunction.

Any adequate theory on the cause of Down's disease, particularly with reference to the maternal age effect must be able to account for several apparently unrelated observations, the effect of maternal age on the haptoglobin phenotypes shown in this section, the high prevalence of thyroid disease and thyroid antibodies in the mothers of Down's subjects (the sex-limited or sex-influenced tendency outlined above), the increased risk of a second affected child subsequent to the birth of a Down's child, etc. The raised thyroid antibodies level is confined chiefly to the younger mothers (18) and Fialkov's studies support the suggestion of the two classes of mothers. Predisposition to Hashimoto's disease is inherited with one or more genes associated (a) with thyroid disease and (b) with early onset of the production of a child with Down's syndrome. Interpopulation differences between the sizes of
the classes A and B will be largely due to differences in
the frequencies of the predisposing genes. According to
the Unified Theory of Growth and Disease (20, 21) idiopathic
autoimmune disorders arise in genetically predisposed persons
as the result of spontaneous somatic gene mutation in mesen-
chymal growth-control stem cells. The mutant stem cell
propagates a 'forbidden clone' (18, 19) of cells which pro-
duce humoral or cellular (but non-immunoglobulin (20, 21) )
autoantibodies. Primary antibodies are better described as
mutant mitotic control proteins (MCP). In its non-mutant
configuration each specific MCP regulates normal growth of a
specific target tissue, whereas the mutant MCP carries out an
autoaggressive attack on the specific tissue it normally
regulated. All tissues external to the central growth
control system are, by definition, target tissues and it has
been predicted that at least one autoaggressive disorder will
be associated (with the appropriate genotype) with each dis-
tinct target tissue (20). Germ cells cannot for any theore-
tical reason be exempt from this generalisation and it has been
 argued that chromosomal non-disjunction usually results from
such an autoaggressive attack on the germ cells (6).

Henderson and Edwards (22) were able to examine oocytes in
mice of different ages, they counted the number of chiasmata
noting their position and also counted the number of univalents (i.e. chromosomes that have no chiasmata, under which circumstance they may fail to pair properly and thus drift apart). They found a decrease in chiasmata count and an increasing frequency of univalents with increasing age. From these observations and from scanty evidence in humans they consider a failure of chiasmata formation at the first meiotic division leads to unequal segregation of the univalents at anaphase. They present two hypotheses to explain the decline in chiasmata formation, in the first the gradients could be present in the foetal ovary at the time of chiasmata formation and these lead to differences in the production of the oocytes. Because the complex dependence of Down's birth on maternal age agrees well with the auto-aggressive theory and because other chromosomal disorders show poorer age dependence. Burch (6) doubts this hypothesis. The alternate theory is that chiasmata may form normally during foetal life but be lost during dictytytone.

Extrinsic factors such as infective agents (23) might promote the separation but the regularity and detailed mathematical form of the frequency: maternal age statistics conflict with this theory that chance infection could directly effect
specific chromosomal non-disjunction at any stage of dicto-
tene or meiosis. Nevertheless, an appropriate infection
often precipitates or exacerbates a latent autoagressive
attack (6) and specific intrinsic factors such as mutant
MCP could conceivably abrogate a potentially dangerous
situation. Goh and Summers (24) have recently demonstrated
a non-viral agent in the plasma of irradiated subjects that
induces chromosomal breaks in normal cultured leucocytes.

In this section two possible factors have been investigated,
the possible change of frequencies associated with paternal
age and possible incompatibility between the foetus and
mother. The former investigation was successful, there does
seem to be a relationship between the frequency of haptoglo-
bin phenotypes in the class A and class B. The
necessary limitations imposed by time and cost has limited
the scope of the incompatibility studies. The whole problem
of detecting such incompatibility has been adequately
summarised by Cohen (25). Reed (26) after a study of several
thousand families had to conclude that "it is important to
assume that selective effects of these blood groups (he
tested a number)......on reproduction are absent or
negligible. A mating type effect on a number of pregnancies
of as much as 5 - 10% of the mean might not have been recog-
nised as a real effect....Studies to data have not tested adequately for the existence of a weak effect.". The sample in this investigation is far too small to detect any possible incompatibility, a much larger sample, together with much more detailed reproductive and mating information would be required to conclusively prove or disprove any maternal/foetal incompatibility involvement. There is little doubt that such incompatibility does exist within the ABO and Rh systems, and this, together with the observations on the haptoglobin system in this series makes interesting the observations that there is considerable interaction between the two systems (27, 28).
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   J. ment. Sci. 22, 161

2. Penrose, L.S. (1932)
   On the Interaction of Hereditary and Environment in the Study of Human Genetics with Special Reference to Mongolian Imbecility.
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3. Jenkins, R.L. (1932)
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4. Penrose, L.S. (1934)
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5. Richards, B.W.R. (1973)
   Mongols and their Mothers.

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*Clin. Genet.* 3, 334


A Note on the Testing of the Hardy-Weinberg Law.

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9. Penrose, L.S. (1934)

A method for the Separating the Relative Aetiological Effects of Birth Order and Maternal Age, with Special Reference to Mongols.

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The Estimation and Significance of the Logarithm of a Ratio of Frequencies.

*Ann. hum. Genet.* 20, 309
   Red Cell Acid Phosphatase, Phosphoglucomutase and Adenylate Kinase.

    Nature 181, 824

    Progr. Allergy 6, 155

    The Causes of Down's Syndrome.

15. Penrose, L.S. (1951)
    Maternal Age in Familial Mongolism.
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    The Distal Triradius on the Hands of Parents and Sibs of Mongols.
    Ann. hum. Genet. 19, 10
17. Richards, B.W. (1972)

Observations on the Mosaic Parents of Mongol Propositi.

J. ment. Defic. Res. 14, 342

18. Burnett, F.M. (1965)

Somatic Mutation and Chronic Disease

Brit. Med. J. 13, 38


A Modern Basis for Pathology.

Lancet 1, 1383


Self and Not Self a Clonal Approach to Immunology.

Quart. Rev. Biol. 40, 252


An Enquiry Concerning Growth, Disease and Ageing.

Oliver Boyd: Edinburgh.


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Nature 218, 22

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*J. Ment. Defic. Res.* 10, 266


Breaks in the Normal Human Chromosomes: Are they Induced by a Transferable Substance in the Plasma of Persons Exposed to a Total Body Radiation.

*Rad. Res.* 35, 171


ABO and Rh Incompatibility 1. Foetal and Neonatal Mortality with ABO and Rh Incompatibility: Some New Interpretations.

*Am. J. hum. Genet.* 22, 414


Research on Blood Groups and Selection from the Child Health and Development Studies, Oakland California. 3. Couple Mating type and Reproductive Performance.

*Am. J. hum. Genet.* 20, 129


Interaction Between the ABO Blood Group and Haptoglobin System.

*Am. J. hum. Genet.* 22, 384


Ann. hum. Genet. 34, 329
SECTION 4

INCREASED RATE OF INFECTION AND ITS POSSIBLE RELATIONSHIP TO CHANGES IN SERUM AND TISSUE PROTEINS
INTRODUCTION

It is generally regarded that Down's syndrome is accompanied by an increase in susceptibility to infections, and deficient antibody formation has been suggested as the possible cause. Several studies on the response of Down's subjects to immunisation has produced conflicting conclusions. Penrose (1) quoting the findings of Donner (2) considered that they showed no differences in this respect, a finding supported by the studies on tetanus immunisation (3, 4) and whooping cough agglutinins (5), although contrary data is available (6). Although an anatomical basis has been postulated for the frequent respiratory infections incurred by Down's subjects (7) it has been shown that both cellular and humoral immune systems differ from those of normal individuals. Impaired delayed hypersensitivity reactions to several antigens have been described (8) whilst some investigators showed that the in vitro response of circulating lymphocytes to phytohaemagglutinin was reduced except at very low concentrations (9). In a study on the lacrymal proteins (10) it was shown that an overall increase in protein concentration in Down's syndrome of an $\alpha_1$-globulin type found only in neonates. These authors suggested that abnormalities of
transport of proteins across cell membranes could be a possible explanation of their finding although similar abnormalities have been shown to occur in tears of normal persons with respiratory infections (11).

An ideal tool for the study of the various aetiological factors in the increase of infection rate in Down's syndrome became available after the initial observation that these subjects exhibited an increased prevalence of the so-called Australia antigen (Au, HAA) or hepatitis associated antigen (12 - 14). It is outside the scope of this introduction to assess the merits of the case for and against the assumption that the HAA protein is part of the original long-incubation (B type) hepatitis virus, and the natural history of this infection has been admirably summarised elsewhere (15). For the purposes of this investigation it will be assumed that the presence of the antigen in the blood of a subject implies that that subject has recently been exposed to the infection. The original observation on the increased frequency of the antigen has been confirmed by a number of subsequent investigations which are summarised in Table 1 below.
## Table 1

<table>
<thead>
<tr>
<th>Date</th>
<th>Reference</th>
<th>Hospital Environment</th>
<th>Down's Subjects Number</th>
<th>% Positive</th>
<th>Control Subjects Number</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1968</td>
<td>14</td>
<td>Large</td>
<td>310</td>
<td>27.7</td>
<td>188</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small</td>
<td>33</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Home</td>
<td>43</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1970</td>
<td>15</td>
<td>Large</td>
<td>130</td>
<td>27.0</td>
<td>299</td>
<td>20</td>
</tr>
<tr>
<td>1971</td>
<td>41</td>
<td>Large</td>
<td>74</td>
<td>31</td>
<td>74</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Small</td>
<td>18</td>
<td>11</td>
<td>79</td>
<td>3</td>
</tr>
<tr>
<td>1972</td>
<td>22</td>
<td>Large</td>
<td>160</td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1973</td>
<td>42</td>
<td>Large</td>
<td>115</td>
<td>20</td>
<td>91</td>
<td>4.4</td>
</tr>
<tr>
<td>1973</td>
<td>43</td>
<td>Large</td>
<td>26</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>This</td>
<td>Investigation</td>
<td>Large</td>
<td>246</td>
<td>28.8</td>
<td>305</td>
<td>3.4</td>
</tr>
</tbody>
</table>

It must be noted that the samples in Table 1 above are not strictly comparable as age and sex differences have been shown to affect the overall frequency of the antigen, with males showing a higher frequency than females and a decrease in frequency in both sexes with increasing age (15).

Although there is an increase in the frequency of the antigen in Down's subjects over their institutionalised peers, this
does not automatically imply an abnormal immune response. It has been suggested that a more likely explanation may be the retention, by Down's subjects, of the agent chronically (16) resulting from some inherited immunological defect, possibly involving impaired lymphocyte function.

Blumberg (21) has proposed an interesting hypothesis that the HAA agent could be construed as a polymorphism with two alleles $A^1$ and $A^2$ with the $A^1:A^1$ genotype open to subclinical persistent infections. If such a gene was located on the trisomic chromosome then there would, by the Hardy-Weinberg law, be an increase in these $A^1:1$ types (albeit as the unusual $A^1:1:2$) at the expense of the other two homozygous forms. There are some indications in the literature on how these mechanisms could be studied. For example treatment of the purified antigen with the detergent Tween 80 showed it to partially dissociate into soluble components which appear to be serum proteins ($\text{IgG, light and heavy chains, complement, beta-lipoprotein, transferrin and albumin}$) (20). Blumberg (21) further proposed that if the HAA acted as a polymorphism then the host protein may not be the same as that of the person it subsequently reinfects, i.e. it acts as an iso-antigen and one could predict circumstances in which antibodies may or may not be produced.
depending on the phenotypes of the recipient and original host, an hypothesis which goes a long way to explain the findings of Hollinger (22). A second suggested line for investigation is found in the studies on the relationship between the ABO system and HAA in normal populations, some of which have shown a relationship to occur (23, 24) although one study failed to support them (25). More recently the possible relationship between HAA and tissue type (HLA system) have proved promising with a proponderence of HLA types W27, W17, HL-A3 and W19 being reported (28) although a more recent study on a larger sample failed to confirm any increase in frequency of 28 alleles at the two HLA loci.

In this Section 53 Down's subjects positive for the antigen will be compared with HAA negative Down's subjects for serum protein levels and for some of the polymorphic frequency distributions.
METHODS

Detection of Hepatitis-Associated Antigen

At the start of this investigation the most practical test available for the presence of the antigen was the counter-electrophoretic method. However, during the course of the investigation a commercial haemagglutination test became available (Hepanosticon: Organon Limited) followed by a second haemagglutination test (Welcome Limited), and although a larger proportion of the data in this investigation is based on these agglutination tests, only some 30 positive patients (i.e. those detected by the less sensitive electrophoresis) could be followed for four years.

The two haemagglutination tests are very similar, with the Organon system based on the agglutination of sheep erythrocytes while the Welcome are based on turkey cells. The former test was used as standard with the latter being employed only as a confirmatory test. The instructions of the manufacturer were carried out: the erythrocytes were suspended in normal saline, a round bottom tube was filled with 500 µl of this suspension and 10 µl of serum added and after thorough mixing incubated at room temperature for three hours, the sedimentation pattern was read by means of an angled mirror with a negative reaction.
being indicated by a sharply outlined ring at the bottom of the tube. All positive tests were re-tested by the Welcome test and by prior absorption on the Hepanosticon absorption kit and re-testing. This absorption step is based on the removal of interfering compounds by fixed sheep cells coated with IgG without anti-HAA activity. A flow chart of the total procedure is given in Fig. 1. This technique has been the subject of a communication (30) in which no false negatives were found in 4960 blood donors, and which showed the technique to be much more sensitive than the counter-electrophoresis, or radial diffusion methods.

**Counterelectrophoresis**

The technique was based on that of Colliford (31), 25ml of a 1% agarose in barbitone:sodium barbiturate buffer (pH 8.8) was poured onto plates 130 x 80mm, and after cooling, wells 2mm diameter were cut in pairs 2mm apart. One well filled with the test serum and the other (cathodic to the first) with specific anti-HAA sera (Welcome Limited). Electrophoresis was carried out at 40mA for 30 minutes at room temperature and a precipitation line mid-way between the two wells at right angles to the current indicated a positive result.
Fig. 1. Flow Chart for the Identification of HAA Positive Sera.

Resuspended reagent in normal saline

- 4 tests 2.2 ml.
- 20 tests 10.5 ml.

Serum or plasma
Shake until homogenous

- 10 micro litres
- 500 micro litres

Shake until homogenous (15-30 secs.)
Leave undisturbed at room temp. (3 hrs.)
Read result

If the result is positive the following absorption procedure must be performed.

1. Resuspend absorbent in saline
2. Absorbent suspension 50 ml centrifuge
   - 10 ml saline per tube
   - 5 min. 1200xg

3. Test fluid 0.010 ml
   - Add 0.10 ml saline
   - Shake
   - Incubate
   - Add 0.30 ml saline
   - Resuspend
   - Centrifuge
   - Sediment supernatant discard

4. 1 hr. 37°C (shake at 30 and 60 min.)
   - 16-20 hrs. 2-8°C

5. Resuspend reagent in saline
   - For 4-test tube 0.45 ml
   - For 20-test tube 2.1 ml
   - 5 min. 1200xg

6. Resuspend reagent
   - 15-30 sec

7. Read pattern
   - After 3 hrs
Serum Alkaline Phosphatase

Serum alkaline phosphatase was estimated at pH 10.5 (0.05 M glycine buffer) using 0.0055 M p-nitrophenyl phosphate as substrate, and the resulting colour estimated at 405nm.

Liver Enzymes

A number of liver enzymes were estimated at 340nm using test kits obtained commercially (Boehringer U.K. : London).
RESULTS

Of the original sample of 256 Down's subjects, 30 were found to be positive by the counterelectrophoresis. These subjects were re-tested at approximately yearly intervals and the data on this is summarised in Table 2 below.

Table 2

<table>
<thead>
<tr>
<th>Year</th>
<th>No. Positive</th>
<th>Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>4+</td>
<td>8 (Strong +)</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>6 (Weak +)</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2. Re-test Data at Yearly Intervals of 30 Down's Subjects Shown to be HAA Positive by Counterelectrophoresis, by Haemagglutination.

As can be seen from Table 2 a considerable number of the positives remained as active carriers up to four years after the original detection. During this time there was no evidence of re-infection by type B virus, but re-infection
Section 4

at sub-clinical levels could not be discounted. Re-testing the sample using the more sensitive haemagglutination test revealed a further 41 cases were shown to be positive making a total of 71 cases (27.7% of the sample). Using the latter technique 304 control samples were made and 10 were found to be positive (3.04%).

Immunoglobulins and HAA Positive Sera

Table 3 compares the four principal immunoglobulins in 40 HAA+ Down's subjects with a number of control subjects and Down's subjects negative for the antigen. Included in Table 3 are the t-tests between the positive Down's and both negative Down's and control groups.
Table 3

<table>
<thead>
<tr>
<th>Test</th>
<th>Sample</th>
<th>No.</th>
<th>Mean (mg/100ml)</th>
<th>SD</th>
<th>T-Tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Down's +</td>
<td>40</td>
<td>1452.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Down's -</td>
<td>66</td>
<td>1384.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>99</td>
<td>1539.0</td>
</tr>
<tr>
<td>IgA</td>
<td></td>
<td></td>
<td>No.</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40</td>
<td>319.4</td>
<td>112.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>66</td>
<td>309.1</td>
<td>140.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>99</td>
<td>272.4</td>
<td>109.0</td>
</tr>
<tr>
<td>HAA+ x HAA-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Down's</td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>1.76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| IgG    |         |     | Down's +        | 42  | 127.0   |
|        |         |     | Down's -        | 82  | 131.0   |
|        |         |     | Controls        | 101 | 132.0   |
| HAA+ x HAA- |
| Down's| 0.37    |
| Controls| 0.54  |

| IgM    |         |     | Down's +        | 40  | 6.9     |
|        |         |     | Down's -        | 62  | 7.9     |
|        |         |     | Controls        | 60  | 3.1     |
| HAA+ x HAA- |
| Down's| 1.02    |
| Controls| 5.95  |

| IgD    |         |     | Down's +        | 40  | 112.2   |
|        |         |     | Down's -        | 66  | 140.6   |
|        |         |     | Controls        | 99  | 109.0   |
| HAA+ x HAA- |
| Down's| 0.41    |
| Controls| 1.76  |

\[^\text{\textsuperscript{+}}p = 0.001\]

Table 3. Comparison of the Four Immunoglobulin Levels in Down's subjects Positive and Negative for HAA, and Control Subjects Negative for HAA.

From the t-tests in Table 3 there is no significant differences between the Down's subjects positive for the antigen and either the negative Down's subjects or the control subjects.
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for IgA, IgG or IgM but a significant increase in IgD levels could be shown with both groups of Down's syndrome when compared with the control groups but not between the two Down's groups.

Liver Enzymes and HAA

In Section 1 of this investigation the elevation of IgD in Down's subjects was noted, and in passing it was postulated that this was probably due to the chronic infectious state of the Down's subjects, and this hypothesis was tested by carrying out a series of specific liver enzyme estimations in the two sub-groups of Down's subjects (i.e. HAA + and HAA -) and control subjects. These enzymes included serum alkaline phosphatase (ALP), lactic dehydrogenase (LDH), isocitric dehydrogenase (ICDH), aldolase (ALD), glutamic-oxaloacetic transaminase (AST), glutamic-pyruvic transaminase (ALT), leucine aminopeptidase (LAP), and glutamic-lactic dehydrogenase (GLDH). The raw data on these tests in the three groups are shown in Table 4, and the t-tests between groups and the correlations with the four immunoglobulins are shown in Tables 5 and 6.
Table 4

<table>
<thead>
<tr>
<th>Test</th>
<th>Controls Mean</th>
<th>SD</th>
<th>Down's Mean</th>
<th>SD</th>
<th>Down's + Mean</th>
<th>SD</th>
<th>Down's - Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>64.9</td>
<td>19.0</td>
<td>58</td>
<td>24.4</td>
<td>56</td>
<td>24.2</td>
<td>59.5</td>
<td>24.6</td>
</tr>
<tr>
<td>LDH</td>
<td>189.2</td>
<td>68.0</td>
<td>165.6</td>
<td>69.0</td>
<td>169.2</td>
<td>64.0</td>
<td>162.2</td>
<td>66.1</td>
</tr>
<tr>
<td>ICDH</td>
<td>5.2</td>
<td>3.3</td>
<td>5.1</td>
<td>2.7</td>
<td>5.0</td>
<td>2.9</td>
<td>5.2</td>
<td>2.9</td>
</tr>
<tr>
<td>ALD</td>
<td>1.01</td>
<td>0.74</td>
<td>1.65</td>
<td>1.0</td>
<td>1.7</td>
<td>1.41</td>
<td>1.55</td>
<td>1.28</td>
</tr>
<tr>
<td>AST</td>
<td>13.6</td>
<td>8.1</td>
<td>15.7</td>
<td>7.0</td>
<td>13.2</td>
<td>8.3</td>
<td>17.7</td>
<td>6.9</td>
</tr>
<tr>
<td>LAP</td>
<td>22.0</td>
<td>6.0</td>
<td>16.6</td>
<td>4.2</td>
<td>18.9</td>
<td>3.9</td>
<td>14.9</td>
<td>4.3</td>
</tr>
<tr>
<td>GLDH</td>
<td>1.18</td>
<td>0.84</td>
<td>0.61</td>
<td>0.38</td>
<td>0.6</td>
<td>0.36</td>
<td>0.6</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Table 4. Comparison of Several Serum Enzyme Levels (mU/ml) in a Control Sample (n = 20) and in a Down's sample (n = 70) with the Down's Sample also Divided into those Positive for HAA (n = 30) and those Negative for the Antigen (n = 40).

The t-tests in Table 5 below show a number of significant deviations from the control group (ALD, ALT, LAP and GLDH) but in all these but ALT the significant deviations were found in both Down's sub-groups. Comparison of the two Down's groups confirmed significant differences in AST, ALT and LAP, but as the deviation of the AST is in favour of the Down's subjects negative for the antigen it was felt that this finding made no contribution to, the problem of liver function and the defective immune response mechanism. The other three significant tests suggest that in both the Down's group there is a significant deviation from the control level but this
deviation is enhanced in the HAA + subjects.

Table 5

<table>
<thead>
<tr>
<th>Test</th>
<th>Controls x</th>
<th>Down's + (Combined)</th>
<th>Down's +</th>
<th>Down's -</th>
<th>Down's + x Down's -</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>0.93</td>
<td>1.12</td>
<td>0.94</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>LDH</td>
<td>1.05</td>
<td>1.04</td>
<td>1.46</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>ICDH</td>
<td>0.11</td>
<td>0.23</td>
<td>0.06</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>ALD</td>
<td>2.10*</td>
<td>2.52**</td>
<td>2.07*</td>
<td>0.70</td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>0.85</td>
<td>0.17</td>
<td>1.93</td>
<td>2.40**</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>2.43**</td>
<td>4.4***</td>
<td>1.99</td>
<td>2.40**</td>
<td></td>
</tr>
<tr>
<td>LAP</td>
<td>3.20***</td>
<td>2.04**</td>
<td>4.74***</td>
<td>4.06***</td>
<td></td>
</tr>
<tr>
<td>GLDH</td>
<td>2.70***</td>
<td>2.70**</td>
<td>2.98***</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. T-Tests between the Three Groups of Data Shown in Table 4.

*P = 0.05, **P = 0.02, ***P = 0.001
The correlation matrix in Table 6 above shows there are no significant relationships between the four immunoglobulins and the various enzyme levels in the control groups, but several correlations do exist in the Down's subjects (underlined). A series of z-transformations were carried out on
these significant correlations and the two Down's groups compared. No significant t-test between the z-transformations between the two Down's groups could be detected. The overall impression gained from the data in Tables 4 - 6 is that there is an increased number of correlations between the liver function tests and the immunoglobulins (particularly IgG and IgD) in the Down's group positive for the antigen compared with that negative for the antigen, but either extended samples or more sensitive techniques would be required to confirm this. It should be pointed out however, that in a matrix such as is illustrated above, a number of chance correlations could be expected to arise. In Table 6 some 96 correlations are listed and by chance 5 significant correlations at the 5% level, 2 correlations significant at the 2% level and one at the 1% level could be expected. The suggestion that the correlations listed in Table 6 as being significant (i.e. underlined) are true and not simply chance observations is strengthened by the observation that with the exception of the one involving LDH and one involving ICDH the majority of significant correlations is limited to the three enzyme systems ALP, AST and ALT.

The relationship between haptoglobin levels and hepatic
Section 4

disorders is now well documented (33) and reported differences in the literature on the frequency of hypohaptoglobinemia probably result from the failure to recognise that the detection methods are crucial in the diagnosis of hypohaptoglobinemia, and that the reduction in haptoglobin levels can vary from time to time depending on the state of hepatic decompensation and the presence of biliary obstruction (32). Storiko has shown (34) that the mean concentrations of haptoglobin in six cases of cirrhosis to be 100mg/100ml (normal mean 160mg/100ml) which is less severe than the has been reported elsewhere (35) but Storiko does report a mean of 41mg/100ml in four cases of cirrhosis with portocaval anastomosis. Similarly the reduction of the Gc protein level in the serum of persons with liver disease has also been reported (36 - 39). The serum levels of these two proteins (Gc and Hp) were shown, for Down's syndrome as a group, to be within normal levels in Section 1 of this investigation. In view of the possible increase in liver abnormalities in those subjects positive for HAA this data has been re-evaluated in Table 7 below.
Table 7

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hp (mg%) Mean</th>
<th>SD</th>
<th>Gc (mg%) Mean</th>
<th>SD</th>
<th>Correlation of Hp with IgA</th>
<th>IgG</th>
<th>IgM</th>
<th>IgD</th>
<th>Correlation of Gc with IgA</th>
<th>IgG</th>
<th>IgM</th>
<th>IgD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>228</td>
<td>104</td>
<td>32.6</td>
<td>8.9</td>
<td>0.28</td>
<td>0.08</td>
<td>0.17</td>
<td>0.06</td>
<td>0.03</td>
<td>0.08</td>
<td>0.16</td>
<td>0.08</td>
</tr>
<tr>
<td>B</td>
<td>232</td>
<td>124</td>
<td>35.9</td>
<td>9.2</td>
<td>0.16</td>
<td>0.21</td>
<td>0.38</td>
<td>0.35</td>
<td>0.02</td>
<td>0.24</td>
<td>0.41</td>
<td>0.13</td>
</tr>
<tr>
<td>C</td>
<td>236</td>
<td>119</td>
<td>34.3</td>
<td>10.4</td>
<td>0.32</td>
<td>0.19</td>
<td>0.24</td>
<td>0.21</td>
<td>0.14</td>
<td>0.31</td>
<td>0.09</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Sample A Control Subjects (n = 20)
Sample B Down's Subjects (HAA -) (n = 20)
Sample C Down's Subjects (HAA +) (n = 20)

T-Tests

<table>
<thead>
<tr>
<th></th>
<th>Hp</th>
<th>Gc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A x B</td>
<td>0.10</td>
<td>1.15</td>
</tr>
<tr>
<td>A x C</td>
<td>0.44</td>
<td>0.55</td>
</tr>
<tr>
<td>B x C</td>
<td>0.10</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Table 7. Means and Standard Deviations of Hp and Gc (mg%) of Control Subjects and Down's Subjects Positive for HAA and Negative for HAA. Also shown are the Correlations of the Hp and Gc levels with the Four Immunoglobulins for the Three Groups and T-Tests Between Groups for Mean Hp and Gc Levels.

The data in Table 7 above failed to reveal any statistical difference in either Hp or Gc levels between the three groups.
(t-tests) nor could any significant correlations between the two proteins and the immunoglobulin be detected in any of the three groups. It is suggested that these findings indicate that there is no lowering of either the Hp or Gc levels due to abnormal liver function which might invalidate the statement in Section 1 of this investigation that these two proteins are within normal limits in Down's syndrome.

HAA and the ABO Locus

Table 7 compares the ABO blood group distribution of 71 Down's subjects positive for HAA and 156 Down's subjects negative for the antigen.

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>AB</th>
<th>0</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>30</td>
<td>7</td>
<td>2</td>
<td>32</td>
<td>71</td>
</tr>
<tr>
<td>-</td>
<td>61</td>
<td>22</td>
<td>3</td>
<td>70</td>
<td>156</td>
</tr>
<tr>
<td>Total</td>
<td>91</td>
<td>29</td>
<td>5</td>
<td>102</td>
<td>227</td>
</tr>
</tbody>
</table>

Comparison of the two groups in Table 8 failed to show any significant change in blood group frequencies ($G = 0.96$). In their original report, Arndt-Hanser et al (23) detected an association in normal subjects between the ABO groups and
Section 4

the presence of the antigen by combining groups A and B
and comparing this with the combined AB and O. When the
data is treated similarly no such significant association
could be detected ($G = 0.02$).

Australia Antigen and Serum Phenotype Frequencies

Table 8 summarises the phenotype frequencies of the three
serum polymorphic systems Gc, Hp and $\beta_2$-glycoprotein 1 in
the control subjects and Down's subjects either positive
or negative for the Australia antigen. The expected fre­
quencies in Table 8 are based on the assumption that the
Hardy-Weinberg law is operative in these three groups.

The data on these three polymorphic systems can be analysed
by carrying out using the Haldane Log ratio test for
heterozygotes between groups and this data will be combined
with similar analysis on a number of red cell polymorphisms
in Table 10.
Table 8

<table>
<thead>
<tr>
<th>System</th>
<th>Pheno-type</th>
<th>Down's HAA + Found</th>
<th>Down's HAA + Expected</th>
<th>Down's HAA - Found</th>
<th>Down's HAA - Expected</th>
<th>Controls HAA- Found</th>
<th>Controls HAA- Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>12 10.3</td>
<td>29 32.9</td>
<td>122 147</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hp</td>
<td>2:1</td>
<td>23 26.1</td>
<td>448 405.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>18 16.6</td>
<td>283 280.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>14 15.1</td>
<td>128 128.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gc</td>
<td>2:1</td>
<td>20 17.7</td>
<td>108 105.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>4 5.3</td>
<td>19 21.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N:N</td>
<td>40 39.2</td>
<td>41 41.9</td>
<td>80 80.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2-Gp</td>
<td>N:D</td>
<td>4 4.8</td>
<td>11 10.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D:D</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Frequency Distributions of Three Serum Polymorphic Systems for Control (HAA-) Subjects and for Down's Subjects Either HAA+ or HAA-

Australia Antigen and Red Cell Polymorphic Frequencies

In Table 9 the frequency distributions of six red cell polymorphic systems for a control group and for two groups of Down's subjects (positive and negative for HAA) are compared. The expected frequencies are based on the Hardy-Weinberg law, but in the case of the acid phosphatase system the expected
Section 4

frequencies are limited to the two most common alleles.

Table 9

<table>
<thead>
<tr>
<th>System</th>
<th>Pheno-type</th>
<th>Down's HAA +</th>
<th>Found</th>
<th>Expected</th>
<th>Down's HAA -</th>
<th>Found</th>
<th>Expected</th>
<th>Controls HAA -</th>
<th>Found</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGT</td>
<td>1:1</td>
<td>26</td>
<td>28.2</td>
<td>112</td>
<td>110.3</td>
<td>238</td>
<td>244.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>23</td>
<td>20.9</td>
<td>66</td>
<td>69.7</td>
<td>159</td>
<td>146.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>4</td>
<td>3.9</td>
<td>13</td>
<td>11.0</td>
<td>15</td>
<td>21.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADA</td>
<td>1:1</td>
<td>45</td>
<td>44.8</td>
<td>165</td>
<td>161.6</td>
<td>374</td>
<td>369.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>8</td>
<td>7.8</td>
<td>22</td>
<td>28.1</td>
<td>40</td>
<td>47.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>0</td>
<td>0.3</td>
<td>4</td>
<td>1.2</td>
<td>4</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACP</td>
<td>A</td>
<td>10</td>
<td>7.8</td>
<td>32</td>
<td>28.2</td>
<td>51</td>
<td>55.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>17</td>
<td>21.4</td>
<td>66</td>
<td>75.0</td>
<td>192</td>
<td>181.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>17</td>
<td>14.8</td>
<td>55</td>
<td>49.7</td>
<td>141</td>
<td>147.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>4</td>
<td></td>
<td>-</td>
<td>8</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BC</td>
<td>3</td>
<td></td>
<td>-</td>
<td>7</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
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<td>10.4</td>
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<td>1</td>
<td>10</td>
<td>10.17</td>
<td>11</td>
<td>10.4</td>
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</tr>
<tr>
<td>ALT</td>
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<td>26</td>
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<td>91</td>
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<td>98</td>
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<td>2:2</td>
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<td>13.0</td>
<td>49</td>
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<td>49</td>
<td>52.7</td>
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</tr>
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</table>

Table 9. Observed and Expected Frequencies of Six Red Cell Phenotype Frequencies in Control Subjects and in Down's Subjects Positive and Negative for HAA.
In the Down's+ subjects in no system did the percentage excess exceed twice the standard error of the estimate and this suggests that no heterozygote advantage could be detected. In the Down's subjects negative for the antigen however there was a slight but significant decrease in heterozygote frequencies in both the ADA and the AK systems, but as these two systems are the ones in which the lowest heterozygote frequencies could be expected and a slight sampling bias would distort...
the final result, it was felt that the significance of this observation is open to some doubt. The excess of the haptoglobin heterozygotes in the control series has been shown previously.

In Section 3 it was possible to show a relationship between the haptoglobin frequencies and maternal age. This relationship was reinvestigated to include the presence and absence of the Australia antigen, as in Table 11.

<table>
<thead>
<tr>
<th>Group</th>
<th>Homozygous</th>
<th>Heterozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Mean</td>
</tr>
<tr>
<td>Down's +</td>
<td>18</td>
<td>33.17</td>
</tr>
<tr>
<td>Down's -</td>
<td>80</td>
<td>35.63</td>
</tr>
</tbody>
</table>

Table 11. Means and Standard Deviations of the Maternal Ages of 193 Down's Subjects Homozygous or Heterozygous for Haptoglobin with and without HAA.

The data in Table 11 failed to reveal any significant relationship between maternal age, haptoglobin phenotype and the presence of HAA.
DISCUSSION

The data in this section has mainly confirmed the observation of a relationship between the frequency of HAA and the Gc phenotype, and the lack of such relationships with the acid phosphatase and alanine aminotransferase systems (26). The only significant deviation from the Hardy-Weinberg law was that reported elsewhere in this investigation in the haptoglobin system for the control group.

No evidence is available that the maintenance of the chronic state of hepatitis type B is associated with any particular phenotype, nor is there any evidence that the Down's subjects not chronic carriers are subject to the protective influences of heterozygosity at least in the systems investigated.
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SECTION 5

CHANGES IN PROTEIN LEVELS AND FREQUENCY DISTRIBUTIONS

RESULTING FROM SELECTION BY MORTALITY
INTRODUCTION

Several studies have been made on the mortality experience of Down's subjects (summarised by Lillienfeld (1)). The life table in general confirm the clinical impression that although the use of antibiotics has decreased the frequency of respiratory infections in infancy and childhood, it still appears that about 25 - 30 per cent of affected Down's subjects die within the first year and about 50 per cent within the first five years (a detailed consideration of the mortality of Down's syndrome is given in the appendix). As the majority of Down's subjects in this investigation are five years or older it was considered as possible that changes in both the levels of serum and plasma proteins and the frequencies of the polymorphic phenotypes may arise as a result of selection through mortality. It was also felt that some useful information might be gained if a group of subjects with a similar pattern of mortality could be compared with the Down's and control groups in this investigation.

Tuberous sclerosis is an autosomal dominant condition but with a varied clinical picture, characterised by mental
retardation, sebaceous adenoma, calcific cerebral deposition and epilepsy. The congenital malformations affect all three embryonic layers with a predisposition to those organs arising from the ectoderm. The frequency of the condition (which hereafter will be referred to as T.S.) is probably in the range of $33 - 44 \times 10^{-6}$ (2, 3) and in those cases of familial appearance, dominant inheritance has been observed with full penetrance of the T.S. gene, but sporadic cases, probably due to fresh mutations appear in at least half the analysed families (3).

Although T.S. can be compatible with a long life and normal intelligence (4 - 11), the course of the disease is often progressive with most subjects living less than 25 years (12) and some 30 per cent die in the first five years and 75 per cent by the twentieth year (8). The comparison of mortality experience is considered in detail in the appendix.

The choice of T.S. to compare with Down's syndrome offers several advantages, in addition to the similar pattern of mortality, both conditions give rise to mental subnormality and thus both groups can be drawn from the larger mental hospitals with similar problems of hygiene, similar regimes
of vaccination etc. Both groups of subjects show similar patterns in the cause of death with infectious disease (particularly respiratory disease) being one of the major reported causes, and finally both groups of subjects are likely to have been subjected to intensive investigations making for a reasonably accurate diagnosis.

In this section the serum and protein levels and the full range of phenotype frequencies in a sample of 54 subjects with T.S. will be compared with the data on the Down's group previously described.

All the methods used to investigate the T.S. subjects are those described in the previous section.
RESULTS

Table 1

<table>
<thead>
<tr>
<th>Immunoglobulin (mg/100ml)</th>
<th>T.S.</th>
<th>Down's</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Mean</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>---</td>
<td>-------</td>
<td>-------</td>
<td>---</td>
</tr>
<tr>
<td>IgA</td>
<td>54</td>
<td>278.8</td>
<td>51.4</td>
</tr>
<tr>
<td>IgG</td>
<td>54</td>
<td>1309</td>
<td>272.0</td>
</tr>
<tr>
<td>IgM</td>
<td>54</td>
<td>222</td>
<td>54</td>
</tr>
<tr>
<td>IgD</td>
<td>54</td>
<td>4.7</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 1. Serum Immunoglobulin Levels in 54 Cases of Tuberous Sclerosis, 119 Cases of Down's Syndrome and 101 Mentally Retarded Controls.

Table 1 compares the levels of the four principal immunoglobulins in the three groups of subjects. Table 2 compares the densitometric data on the serum proteins and serum glycoproteins of the three groups of subjects and Table 3 compares a number of serum and plasma proteins (radial diffusion) of the three groups. The analysis of the data in Tables 1-3 are shown in Table 4.

One of the T.S. subjects was found to exhibit an additional band of protein in the beta-gamma region on cellulose acetate electrophoresis, which was subsequently shown to be an IgG
Section 5

melanoma protein, and the protein fractionation data on this subject were deleted from Table 2.

<table>
<thead>
<tr>
<th>Protein (g/ml)</th>
<th>T.S.</th>
<th>Down's</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Albumin</td>
<td>53</td>
<td>4.28</td>
<td>0.52</td>
</tr>
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<td>$\alpha_1$-globulin</td>
<td>53</td>
<td>0.22</td>
<td>0.74</td>
</tr>
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<td>$\alpha_2$-globulin</td>
<td>53</td>
<td>0.90</td>
<td>0.12</td>
</tr>
<tr>
<td>$\beta$-globulin</td>
<td>53</td>
<td>1.11</td>
<td>0.26</td>
</tr>
<tr>
<td>$\gamma$-globulin</td>
<td>53</td>
<td>1.28</td>
<td>0.46</td>
</tr>
<tr>
<td>Glycoprotein (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>54</td>
<td>14.8</td>
<td>4.7</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>54</td>
<td>42.1</td>
<td>6.4</td>
</tr>
<tr>
<td>Beta</td>
<td>54</td>
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<td>2.8</td>
</tr>
<tr>
<td>Gamma</td>
<td>54</td>
<td>18.9</td>
<td>6.1</td>
</tr>
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</table>
### Table 3

<table>
<thead>
<tr>
<th>Protein (mg/ml)</th>
<th>T.S.</th>
<th>Down's</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mean</td>
<td>SD</td>
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<tr>
<td>Fibrinogen</td>
<td>54</td>
<td>303.22</td>
<td>73.7</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>54</td>
<td>15.0</td>
<td>4.4</td>
</tr>
<tr>
<td>$\alpha_2$-antithrombin</td>
<td>54</td>
<td>33.4</td>
<td>10.2</td>
</tr>
<tr>
<td>Gc Proteins</td>
<td>54</td>
<td>29.4</td>
<td>7.6</td>
</tr>
<tr>
<td>$\beta$-lipoproteins</td>
<td>54</td>
<td>518.0</td>
<td>137.0</td>
</tr>
<tr>
<td>$\alpha_1$-antitrypsin</td>
<td>54</td>
<td>279.0</td>
<td>57.0</td>
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<td>$\beta_1$-A-globulin</td>
<td>54</td>
<td>94.8</td>
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<td>Transferrin</td>
<td>54</td>
<td>192.3</td>
<td>79.0</td>
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<td>$\alpha_1$-acid Glycop.</td>
<td>54</td>
<td>84.5</td>
<td>41.6</td>
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<td>Haptoglobin</td>
<td>54</td>
<td>79.1</td>
<td>19.8</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
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<td>37.4</td>
<td>7.8</td>
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<td>$\alpha_2$-HS-glycopr.</td>
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<td>52.4</td>
<td>17.9</td>
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<td>Prealbumin</td>
<td>54</td>
<td>31.4</td>
<td>15.1</td>
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<td>$\alpha_2$-macroglob.</td>
<td>54</td>
<td>347.3</td>
<td>62.1</td>
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<tr>
<td>Hemopexin</td>
<td>54</td>
<td>119.1</td>
<td>19.7</td>
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### Section 5

**Table 4**

<table>
<thead>
<tr>
<th>Protein</th>
<th>T.S x Down's T.S.</th>
<th>T.S x Controls</th>
<th>Down's T.S x Controls</th>
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</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>0.013</td>
<td>0.57</td>
<td>0.58</td>
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<tr>
<td>Plasminogen</td>
<td>2.27*</td>
<td>4.6***</td>
<td>1.05</td>
</tr>
<tr>
<td>$\alpha_2$-antithrombin</td>
<td>0.0</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Gc Proteins</td>
<td>2.75**</td>
<td>2.5*</td>
<td>0.9</td>
</tr>
<tr>
<td>$\beta$-lipoprotein</td>
<td>3.5**</td>
<td>5.5***</td>
<td>1.8</td>
</tr>
<tr>
<td>$\alpha_1$-antitrypsin</td>
<td>9.3***</td>
<td>2.4*</td>
<td>1.2</td>
</tr>
<tr>
<td>$\beta_1$-A-globulin</td>
<td>0.73</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Transferrin</td>
<td>4.47***</td>
<td>4.8***</td>
<td>0.38</td>
</tr>
<tr>
<td>$\alpha_1$-acid-glycopr.</td>
<td>2.9**</td>
<td>2.2*</td>
<td>7.2***</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>8.8***</td>
<td>8.6***</td>
<td>-</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>2.1*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\beta_1$-E-globulin</td>
<td>4.1***</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\beta_2$-glycoprotein</td>
<td>10.7***</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\alpha_2$-HS-glycopr.</td>
<td>0.45</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prealbumin</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$\alpha_2$-macroglobulin</td>
<td>14.5***</td>
<td>4.2**</td>
<td>-</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>0.63</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IgA</td>
<td>3.5**</td>
<td>0.67</td>
<td>3.5***</td>
</tr>
<tr>
<td>IgG</td>
<td>3.3**</td>
<td>7.4***</td>
<td>0.3</td>
</tr>
<tr>
<td>IgM</td>
<td>10.3***</td>
<td>10.6***</td>
<td>1.6</td>
</tr>
<tr>
<td>IgD</td>
<td>1.3</td>
<td>2.3*</td>
<td>7.3***</td>
</tr>
</tbody>
</table>
The analysis of the data in Tables 1-3 is shown in Table 4. In several instances control data was not obtained as the Down's data was obviously within normal limits and the cost of such investigations for the sake of completeness was not justified. In two instances where control data was not available the T.S. subjects significantly differed from the Down's subjects (haptoglobin, $t = 8.6, P = 0.001$ and $\alpha_2$-macroglobulin $t = 4.2, P = 0.01$). Comparison of the T.S.
levels of these two proteins with normal reported data (13) showed the haptoglobin to be significantly lowered ($P = 0.01$) and the $\alpha_2$-macroglobulin to be significantly raised ($P = 0.001$). The data in Table 4 although illuminating is still open to severe mis-interpretation, what is being looked for are changes in the Down's proteins when compared with the control subjects which are repeated in a comparison of T.S. and control subjects. The comparison of the Down's and T.S. subjects, when such a comparison yields a significant difference, is much less interesting although it could be argued that in some instances this could be interpreted as the genetic abnormality exerting a pressure contrary to the mortality effect in one group of subjects but not in the other. Only in the two fractions $\alpha_1$-acid glycoproteins and IgD are concordant abnormalities found and it is doubtful if either of these are indicative of selection by mortality. As has been shown previously, the IgD elevation in the Down's group is probably the result of the chronic infectious state. The elevation of the $\alpha_1$-acid glycoprotein is much more difficult to interpret. This protein is known to be raised in conditions leading to an inflammatory state, but in these circumstances it is usually accompanied by elevations in such fractions as the $\alpha_1$-antitrypsin and haptoglobin (14), all three of which
are elevated in the T.S. sample but not the Down's sample. In those cases of increased glomerular permeability, a common feature is albuminuria often accompanied by the presence in urine of $\alpha_1$-acid glycoprotein. The results suggest that the reduction in albumin levels in the Down's group is not due to increased renal filtration but is specific to Down's syndrome. Whether the elevation of the $\alpha_1$-acid glycoprotein is due to specific changes arising from selection by mortality, or secondary to the chronic infectious state is still in doubt but the weight of the evidence favours the latter. The other features of serum proteins in Down's group are not repeated in the T.S. group and this strengthens the suggestion that they are not due to selection by mortality but due to the syndrome per se. Table 5-8 summarise the frequency distributions and analysis of the data on serum and red cell polymorphic systems, and compares them with the data reported in Section 2.
<table>
<thead>
<tr>
<th>System</th>
<th>Phenotype</th>
<th>Frequencies</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine deaminase</td>
<td>1:1</td>
<td>49</td>
<td>374</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>( n = 54 )</td>
<td>418</td>
<td></td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>1:1</td>
<td>53</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>1</td>
<td>11</td>
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<td></td>
<td>2:2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>( n = 54 )</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Phosphoglucomutase I</td>
<td>1:1</td>
<td>29</td>
<td>238</td>
</tr>
<tr>
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<td>2:1</td>
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<td>1:1</td>
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<tr>
<td>Phosphogluconate dehydrogenase A</td>
<td>52</td>
<td>167</td>
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<td></td>
<td>AB</td>
<td>2</td>
<td>11</td>
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<tr>
<td></td>
<td>B</td>
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<td>0</td>
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<tr>
<td></td>
<td>( n = 54 )</td>
<td>178</td>
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<tr>
<td>Acid phosphatase</td>
<td>A</td>
<td>5</td>
<td>51</td>
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<td></td>
<td>AB</td>
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<td>141</td>
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**Table 5.** Frequencies of 6 Red Cell Polymorphic Systems in 54 Subjects with Tuberous Sclerosis Compared with Mentally Retarded Controls.
### Table 6

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<tr>
<th>System</th>
<th>Phenotype</th>
<th>Frequencies</th>
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<td></td>
<td></td>
<td>tuberous</td>
<td>sclerosis</td>
<td>Z²</td>
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<tr>
<td>Haptoglobin</td>
<td>1:1</td>
<td>7</td>
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<td>22</td>
<td>448</td>
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<tr>
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<td>2:2</td>
<td>25</td>
<td>288</td>
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<td></td>
<td>n = 54</td>
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<td>198</td>
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<td></td>
<td>Pp2</td>
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<td></td>
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<td>N:N</td>
<td>52</td>
<td>337</td>
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<tr>
<td></td>
<td>D:N</td>
<td>2</td>
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<td>3.06</td>
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<tr>
<td></td>
<td>D:D</td>
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<td>1</td>
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<td></td>
<td>n = 54</td>
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<td></td>
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<tr>
<td>Gc protein</td>
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<td>128</td>
<td>50.6*</td>
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<tr>
<td></td>
<td>2:1</td>
<td>42</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:2</td>
<td>8</td>
<td>19</td>
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<td></td>
<td>n = 54</td>
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<tr>
<td>Leucine aminopeptidase</td>
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<td>48</td>
<td>80</td>
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<td></td>
<td>21</td>
<td>12</td>
<td>52</td>
<td>80</td>
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<tr>
<td></td>
<td>22</td>
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<td>n = 54</td>
<td>83</td>
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* P = 0.001.

### Table 7

<table>
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<tr>
<th>System</th>
<th>Deviation from the Hardy-Weinberg law (T.S.)</th>
<th>Haldane's Log ratio test</th>
<th>Gene frequencies T.S. group estimate S.E.</th>
<th>controls estimate S.E.</th>
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<td>Adenosine deaminase</td>
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<td>0.041 0.5</td>
<td>ADA¹ 0.99 0.01</td>
<td>0.942 0.01</td>
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<td>ADA² 0.01</td>
<td>0.058</td>
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<td>Adenylate kinase</td>
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<td>0.0871 0.104</td>
<td>AK¹ 0.99 0.01</td>
<td>0.96 0.01</td>
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<td></td>
<td>AK² 0.01</td>
<td>0.04</td>
</tr>
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<td>Phosphoglucomutase 1</td>
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<td>0.173 0.29</td>
<td>PGM¹ 0.73 0.043</td>
<td>0.77 0.015</td>
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<td></td>
<td></td>
<td></td>
<td>PGM² 0.27</td>
<td>0.23</td>
</tr>
<tr>
<td>Glutamic-pyruvic</td>
<td>1.56</td>
<td>0.051 0.33</td>
<td>GPT¹ 0.414 0.051</td>
<td>0.46 0.026</td>
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<tr>
<td>transaminase</td>
<td></td>
<td></td>
<td>GPT² 0.585</td>
<td>0.540</td>
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<tr>
<td>Phosphogluconate</td>
<td>0.04</td>
<td>0.358 0.78</td>
<td>PGDA¹ 0.98 0.013</td>
<td>0.97 0.01</td>
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<td>dehydrogenase</td>
<td></td>
<td></td>
<td>PGDA² 0.02</td>
<td>0.03</td>
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<tr>
<td>Acid phosphatase</td>
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<td>0.128 0.28</td>
<td>Ac⁴ 0.352</td>
<td>0.374</td>
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<td></td>
<td></td>
<td>Ac⁵ 0.601 0.002</td>
<td>0.602 0.017</td>
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<td></td>
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<td>Ac⁶ 0.047</td>
<td>0.024</td>
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</tbody>
</table>

**Table 7. Analysis of the Data on the Red Cell Polymorphisms Shown in Table 5.**
Of the eleven polymorphisms described in Tables 5 and 6 a significant deviation could be shown only in the Gc proteins, which group of proteins also showed a significant deviation from the Hardy-Weinberg Law (Table 8), and a significant difference in the ratio of homozygotes to heterozygotes by the log ratio test.

The only other point of note is the slightly significant log ratio test for the haptoglobin system.
DISCUSSION

The approach selected in this section for testing for selection by mortality by using a group of subjects with similar high rates of mortality and similar patterns of cause of mortality can be criticised on the grounds of non-comparability on purely genetic grounds, one group being a single dominant gene defect and the other a multigene (chromosomal) defect. Additionally, the expression of the two genetic defects are totally dissimilar with both renal and hepatic defects in the T.S. group adding complications to the interpretation of the data. However, failing a long-term follow-up investigation of a number of Down's children from birth, an indirect method such as outlined here is essential.

The information given by the quantitative estimations of a number of serum and plasma proteins is little, but on the whole tend to suggest that in the quantitative aspects of these proteins mortality plays very little part. The phenotype distributions of the T.S. group, however, have proved more enlightening. These were found to be abnormal in the two serum systems haptoglobin and Gc proteins, both systems which have proved to be of considerable interest in the pathology
The observations on these two systems in T.S. is hitherto unreported and will require further follow-up studies which are outside the scope of this investigation. However, considering the observation of a parental age effect on the haptoglobin frequencies in Down's syndrome, it would be interesting to collect a sufficiently large sample of T.S. to carry out a similar study. In the relevant section on parental age, Burch (15) suggested a unified law for autoimmune (autoaggressive) disorders, and it is interesting to speculate whether or not the similarity in the Gc and Hp systems between the two genetic disorders in this section gives some hint of a similar (if not the same) unified law for genetic defects.

The finding of an abnormal Gc distribution in the T.S. group strengthens the suggestion made earlier that the abnormal distribution in Down's syndrome is not due to the loci on the trisomic chromosomes but some other selection, such as mortality, is operating.
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SECTION 6

THEORETICAL STUDY ON THE APPLICATION OF POLYMORPHIC FREQUENCIES TO GENE LOCATION ON A TRISOMIC CHROMOSOME
INTRODUCTION

There is already an extensive literature on the application of the phenotype shift to the investigation of the possible location of the blood group system on the trisomic 21st chromosome in Down's syndrome, and limitations on the grounds of chance have been pointed out by Lawler (4) when applied to the use of blood group phenotype shifts.

Theoretical models for the distortion of the phenotype ratios have, in part, been established (1, 3, 5), with the latter two studies providing models for the shift in the case of a single pair of alleles showing dominance, lack of dominance and dosage effects assuming that the non-disjunction occurred at the first meiotic division. It has, however, been pointed out (4) that this concept becomes more complex if the non-disjunction occurs at the second meiotic division or post-zygotically. Goodman (6) extended the model to include crossing-over, the possible admixture of first and second meiotic non-disjunction and post-fertilisation non-disjunction assuming that these were independent of parental genotypes. For purely theoretic reasons, the application of electrophoretic techniques to the investigation of the phenotype shift
should hold considerable advantages. In these techniques it is the structural gene which is being studied through its direct product, eliminating (with some reservations which will be dealt with below) the difficulties arising from dominance and dosage effects. In addition to eliminating those problems, the technique will allow the investigator to increase the number of potential gene markers. It has been estimated that some 30% of all genes will exhibit polymorphism (7) although there is no evidence that the electrophoretic mobilities of the aberrant forms will always differ sufficiently for correct typing. One of the major disadvantages of the electrophoretic approach is the possible incorrect assessment of trisomic heterozygotes, i.e. with a two allele system it might not be possible to differentiate between, say AB and ABB.

Development of a Model for use with Electrophoresis

The model outlined by Goodman (6) for a two allele system is adequate (if failure to differentiate between heterozygotes is taken into account).
### Table 1

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<td>—</td>
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</tr>
<tr>
<td>AA × AB</td>
<td>2pq</td>
<td>pq</td>
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</tr>
<tr>
<td>AA × AC</td>
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Table 2

Substitution of $k = c + 2t - 3ct$ in the frequencies given in Table 1

<table>
<thead>
<tr>
<th>Mating</th>
<th>$f$</th>
<th>AAA</th>
<th>AAB</th>
<th>AAC</th>
<th>ABA</th>
<th>ABC</th>
<th>ABC</th>
<th>BBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA × AA</td>
<td>$p_1$</td>
<td>$p_1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>AA × AB</td>
<td>$2p_1q$</td>
<td>$p_1q$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA × AC</td>
<td>$2p_1r$</td>
<td>$p_1r$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA × BB</td>
<td>$p_1^2q^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA × CC</td>
<td>$p_1^2r^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB × AA</td>
<td>$2pq$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$(2k)p_q$</td>
</tr>
<tr>
<td>AB × AB</td>
<td>$4p_2q^2$</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>AB × AC</td>
<td>$4p_2qr$</td>
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<tr>
<td>AB × BB</td>
<td>$2p_2q^2$</td>
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<tr>
<td>AC × AB</td>
<td>$2p_2qr$</td>
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<tr>
<td>AC × AC</td>
<td>$4p_2r^2$</td>
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<tr>
<td>AC × BB</td>
<td>$2p_2r^2$</td>
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</tr>
<tr>
<td>AC × CC</td>
<td>$2p_2r^2$</td>
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<tr>
<td>BC × AA</td>
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<tr>
<td>BC × AB</td>
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<td>BC × AC</td>
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<tr>
<td>BC × BB</td>
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<tr>
<td>BC × CC</td>
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<tr>
<td>CC × AA</td>
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<td></td>
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<tr>
<td>CC × AB</td>
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<td></td>
</tr>
<tr>
<td>CC × AC</td>
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</tr>
<tr>
<td>CC × BB</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC × BC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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But it fails to take into account more complex systems such as a triallelic system like acid phosphatase or a system with a 'silent' allele like haptoglobin.

Three Alleles at one Locus

Consider three alleles A, B and C at one locus on the trisomic chromosome with frequencies p, q and r where \( p + q + r = 1 \). If \( c \) is the frequency of crossing over and \( t \) is the frequency of second meiotic division non-disjunction the heterozygous parents will produce non-disjunctional gametes in the proportions:

<table>
<thead>
<tr>
<th>Gamete</th>
<th>1st Division</th>
<th>2nd Division</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA, BB, CC</td>
<td>((1 - t) (\frac{1}{2}c))</td>
<td>(t(\frac{1}{2} - \frac{1}{2}c))</td>
</tr>
<tr>
<td>AB, BC, AC</td>
<td>((1 - t) (1 - \frac{1}{2}c))</td>
<td>(ct)</td>
</tr>
</tbody>
</table>

The results of such 36 possible matings arising from these gametes are given in Table 1.

If, as suggested by Goodman (4) these resulting mating frequencies are simplified by substituting \( k = c + 2t - 3ct \) then the frequencies simplify to those shown in Table 2. The sum totals of the various trisomic frequencies from Table 2 become:
But, as has been pointed out some of these may be indistinguishable in which case the frequencies above will become:

Unfortunately, the variant ABC will vary in mobility dependent on the mobility of the three constituent electrophoretic bands and may be indistinguishable from any of the other heterozygous forms.

**Post-Fertilisation Effects.**

If $m$ is the proportion of trisomics whose non-disjunction arises after fertilisation, then the frequencies of the above phenotypes become:
If a triallelic system such as acid phosphatase in which the normal (disomic) population is well established i.e. \( p = 0.36, q = 0.59 \) and \( r = 0.05 \) for an English population (8) then these can be substituted in the general case (case 1 Table 3) and it should be possible to obtain a general equation involving a chi square test between the normal (disomic) population and the theoretical trisomic population in terms of \( N \) (number investigated), \( k \) and \( m \) i.e.

\[
\frac{\chi^2}{N} = \sum \frac{(p^2 - (p^2m + (pq + \frac{1}{2}Kp^2(1-p)(1-m))^2)}{p^2} + \frac{(2pq - (2pq(1-r)(1-\frac{1}{2}K) + Kpq)(1-m))^2}{2pq}
\]

For a significant shift to have occurred then for \( P = 0.05 \) at \( df = 6 \) then \( \chi^2, 11.0 \). So for various values of \( k \) and \( m \) it should be possible to derive a series of curves involving \( N \)
for that particular enzyme. For example in the red cell acid phosphatase with p, q and r of the value shown above and $x^2 > 11.0$ the above equation becomes:

$$
\begin{align*}
\chi^2_{k=0} &= 0.053m^2 - 0.242m + 0.184 \\
\chi^2_{k=0.5} &= 0.180(1-m)^2 \\
\chi^2_{k=1.0} &= 0.166(1-m)^2 \\
\chi^2_{k=1.5} &= 0.133(1-m)^2
\end{align*}
$$

These equations for values of m from 0 to 1.0 will give a series of curves as shown in Fig. 1 below.

![Diagram showing series of curves relating the size of a triploid population required to establish a significant phenotype shift under differing conditions of crossing-over, post-fertilisation non-disjunction and 2nd meiotic division non-disjunction.](image-url)
Section 6

Bi-Allelic System

Goodman has derived the general case for a bi-allelic system with alleles A and B:

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>$p^3 + \frac{1}{2}pq^2k$</td>
</tr>
<tr>
<td>AAB</td>
<td>$3p^2q + \frac{1}{2}pqk(1 - 3p)$</td>
</tr>
<tr>
<td>ABB</td>
<td>$3pq^2 + \frac{1}{2}pqk(1 - 3q)$</td>
</tr>
<tr>
<td>BBB</td>
<td>$q^3 + \frac{1}{2}pq^2k$</td>
</tr>
</tbody>
</table>

Combining the two heterozygote frequencies as before then a chi-square equation can be obtained as before:

$$x^2 = \frac{1}{N} pq (1 - m)(1 - \frac{1}{2}k)^2$$

which will give a series of curves such as are shown in Fig. 2.
Three Alleles - One 'Silent'

The theoretical frequencies derived in Table 2 would still hold, but with a silent gene it may not be possible to differentiate between, for example AAA and either A00 or AAO, or between BBB and BBO or B00 (assuming the C allele was silent). Similarly ABC becomes ABO and will be indistinguishable from AB.
Section 6

Taking into account these possibilities and using the notation as above, the frequencies of the tri-allelic system ABO where O is silent become:

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO (AOO)</td>
<td>$p^2m+(1-m)(3pq^2+\frac{1}{2}kpr(1-3r))$</td>
</tr>
<tr>
<td>AA (AAB+ABO)</td>
<td>$p^2m+prm+(1-m)(p^3+3pq^2)(1-\frac{1}{2}k)+(\frac{1}{2}kpr(p+i))$</td>
</tr>
<tr>
<td>AD (AAB+ABO)</td>
<td>$2pqm+(1-m)((1-r)(3pq)-(1-3r)(\frac{1}{2}kpr))$</td>
</tr>
<tr>
<td>BO (BOO)</td>
<td>$prn+(1-m)(3qr^2+\frac{1}{2}kqr(1-3r))$</td>
</tr>
<tr>
<td>BB (BBB+BBO)</td>
<td>$q^2m+qrm+(1-m)((1-\frac{1}{2}k)(q^2+3q^2r))$</td>
</tr>
<tr>
<td>OO (OOO)</td>
<td>$r^2m+(1-m)(r^3+\frac{1}{2}kr^2(1-r))$</td>
</tr>
</tbody>
</table>

Application of the Theoretical Model

Table 3 below shows the general and selected cases for a three allele system with the locus on the trisomic chromosome. Cases 4 and 6 are indistinguishable, but has been pointed out elsewhere (6) these, on a priori grounds, are most unlikely. Case 2 is the simplest with no crossing over, all non-disjunction occurring at the first meiotic division again on a priori grounds most unlikely. Unfortunately it is exactly these conditions that most investigators have assumed prior to comparing a disomic with a trisomic population. Applying the above derived equations it should be possible to arrive at a more realistic evaluation of the data and also to obtain for any gene frequencies an estimate of the minimum size of an experimental group required before significant differences can be established.

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Use of Multiple Gene Frequencies

Consider two bi-allelic genes, one of which is presumed to be located on the trisomic chromosome, AA(p²), AB(2pq) and BB(q²) and CCC/CDD/CCD/DDD (C = r, D = s); the frequencies of the various combinations are shown in Table 4. Also shown in Table 4 are the frequencies if both gene loci are located on the trisomic chromosome (in italics).
### Table 4

<table>
<thead>
<tr>
<th></th>
<th>CCC</th>
<th>CCD</th>
<th>CDD</th>
<th>DDD</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p^2 )</td>
<td>( p^2 )</td>
<td>( p^2 )</td>
<td>( p^2 )</td>
<td>( p^2 )</td>
</tr>
<tr>
<td>( q^2 )</td>
<td>( q^2 )</td>
<td>( q^2 )</td>
<td>( q^2 )</td>
<td>( q^2 )</td>
</tr>
</tbody>
</table>

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>( (p^2+YrsK)(1-m))</td>
<td>( (3r^2+YrsK)(1-3r)(1-m)+ram )</td>
<td>( 3r^2+YrsK)(1-3r)(1-m)+ram )</td>
<td>( (3r^2+YrsK)(1-m)+\theta/m )</td>
<td>( (3r^2+YrsK)(1-m)+\theta/m )</td>
</tr>
</tbody>
</table>

**Derived equations for the frequencies of two genes, one of which is located on the trisomic chromosome. In italics are the frequencies if both genes were located on non-trisomic chromosomes.**
Section 6

From Table 4 it is possible to derive an equation to establish the minimum number of tests to confirm that the gene is located on the trisomic chromosome:

\[ \frac{X^2}{N_1} = 2(1 - m)^2 \left( r^2 + \frac{1}{2} rs k \right)^2 + rs \left( \frac{1}{2}(1-k)^2 \right) \]  

for \( df = 8, \ P = 0.05 \) when \( x^2 = 15 \)

Comparison of the above equation with that of a single gene analysis:

\[ \frac{X^2}{N_2} = \frac{1}{2} rs \left( 1 - m \right)^2 \left( 1 - k \right)^2 \]  

for \( df = 2, \ P = 0.05, \ x^2 = 6 \).

It is of interest to note that in equation 1 above, the frequencies of the disomic chromosome are eliminated and only the trisomic chromosome contributes to the final value of \( N_1 \).

With the two equations for any value of gene frequencies it is possible (using a limiting value of \( k \) and \( m \)) to establish \( N_1 \) and \( N_2 \). For example for the Gc proteins with \( r = 0.7 \), if \( k = 1.5 \) and \( m = 0 \) then \( N_1 \) (two genes) = 1278 and \( N_2 \) (one gene) = 932.
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Selection of Limiting Values of $k$ and $m$

In the preceding derivations and curves a limiting value of $k = 1.5$ has been taken. To apply the derived equations some limiting value must be accepted but this need not be an arbitrary value. There is some evidence in the reported literature on this problem.

Lejeune (9) has shown that if the effect of crossing over is ignored, and that if the gene products are quantitative and additive then for a bi-allelic system with $AA(p^2)$, $AB(2pq)$ and $BB(q^2)$ it follows that the expected mean for the gene product would be $2(pA + qB)$ with a variance $2pq(A - B)^2$ and thus the ratio $V/M$ for normals would be:

$$\frac{2pq(A - B)^2}{2(pA - qB)}$$

For trisomics with non-disjunction occurring at the first meiotic division the frequency of the phenotypes would be given by the expansion of $(p + q)^3$ with a mean $3(pA - qB)$ and a variance $3pq(A - B)^2$, i.e. for trisomics the ratio $V/M$ would be:

$$\frac{3pq(A - B)^2}{3(pA + qB)}$$

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i.e. if the non-disjunction occurred at the first meiotic division the ratio of $V/M$ would not be changed. If the non-disjunction occurred at the second meiotic division the mean would be:

$$3(pA + qB) \text{ and } V = 5pq(A - B)^2$$

i.e. the value of $V/M$ for the trisomics would be $5/3$ times higher than the normal disomic counterpart.

Lejeune claims to have analysed data on alkaline phosphatase, galactose uridyl transferase and urinary kinurenine and shown the ratio $V/M$ to be significantly higher in the Down's group pointing to a second division error with $t \to 1.0$ in which case $m \to 0$. The effect of the crossing over will be to reduce the increase in the value of the $V/M$ ratio. Thus with $k = c + 2t - 3ct$ then the overall limiting case will be in the region of $k = 1.5$ when $m = 0.1$. This estimate finds confirmation in the analysis of blood group data by Goodman (6) who suggested that non-disjunction arose post-zygotically or during second meiotic division with $k = 1.31$ with an upper limit of $c = 0.35$. 

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Value of a Mathematical Model

The usefulness of a mathematical model will depend on its ability to predict possible mechanisms or in its use to correct the design of experimental procedures. Reverting to Fig. 2 above, if we assume the limiting values of $k = 1.5$ and $m = 0.1$ then for any gene frequencies it is possible to establish the number of Down's subjects required to establish a phenotype shift.

In Table 5 the minimum number of Down's subjects are shown for eleven gene systems of varying origins and it will be seen that much greater numbers than has been hitherto suspected would be required to detect the phenotype shift by a simple chi square test, numbers that have never been achieved in any investigation (including this one).

This lack of statistical power of the chi square test has led to the use, throughout this investigation of the Haldane log ratio test, the validity of which will be tested in the next paragraph.
Section 6

Table 5

<table>
<thead>
<tr>
<th>Polymorphic system</th>
<th>Source</th>
<th>Frequency of more common allele</th>
<th>Minimum number of subjects required to establish a phenotype shift</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>k=1.5 m=0.1 k=1.0 m=0</td>
<td></td>
</tr>
<tr>
<td>Group-specific proteins</td>
<td>Serum</td>
<td>0.71</td>
<td>1105</td>
<td>233</td>
</tr>
<tr>
<td>Haploglobin</td>
<td>Serum</td>
<td>0.618</td>
<td>1020</td>
<td>202</td>
</tr>
<tr>
<td>Phosphoglucomutase - A</td>
<td>R.B.C.</td>
<td>0.74</td>
<td>1232</td>
<td>249</td>
</tr>
<tr>
<td>Phosphoglucomutase - B</td>
<td>R.B.C.</td>
<td>0.76</td>
<td>1299</td>
<td>263</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>R.B.C.</td>
<td>0.94</td>
<td>4202</td>
<td>851</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>R.B.C.</td>
<td>0.955</td>
<td>5517</td>
<td>1115</td>
</tr>
<tr>
<td>Amylase - 1</td>
<td>Saliva</td>
<td>0.96</td>
<td>6750</td>
<td>1250</td>
</tr>
<tr>
<td>Amylase - 2</td>
<td>Pancreas</td>
<td>0.355</td>
<td>5517</td>
<td>1116</td>
</tr>
<tr>
<td>β-phosphogluconate dehydrogenase</td>
<td>R.B.C.</td>
<td>0.907</td>
<td>7427</td>
<td>1005</td>
</tr>
<tr>
<td>Glutamate-pyruvate transaminase</td>
<td>R.B.C.</td>
<td>0.504</td>
<td>160</td>
<td>192</td>
</tr>
<tr>
<td>Pepsinogen</td>
<td>Urine</td>
<td>0.62</td>
<td>1019</td>
<td>204</td>
</tr>
</tbody>
</table>

Testing for Phenotype Shift Using Log Ratio Test.

If out of N affected persons H had characteristic a and K had character b, and in n unaffected persons the corresponding frequencies were h and k then the log test is given by:

\[
\ln \left( \frac{2H + 1}{2h + 1} \cdot \frac{2K + 1}{2k + 1} \right)
\]

with a variance \( \frac{1}{H} + \frac{1}{K} + \frac{1}{h} + \frac{1}{k} \)

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From the equations derived above in a population of \( N \) \( \text{trisomics} \) the number of heterozygotes (\( AAB + ABB \)) (bi-allelic system) would be:

\[
N( (1 - m) (pq(3 - \frac{1}{2}) + 2pqm) )
\]

and the number of homozygotes (\( AAA + BBB \)) would be:

\[
N( (1 - m) (p^3 + q^3 + \frac{1}{2}pqk) + m (1 - 2pq) )
\]

and for the control (\( \text{disomic} \)) the corresponding frequencies would be:

\[
n(2pq) \text{ and } n(p^2 + q^2).
\]

Applying these four equations to the Haldane log ratio test

\[
\frac{1}{\text{variance}} (2N((p^2+q^2+\frac{1}{2}pqk)(1-m)+m(1-2pq)) + 1)(4pq(1-m)(3-\frac{1}{2}k+2pqm)+1)
\]

These two equations, although appearing unwieldy, enable the investigator to predict for any number of controls and \( \text{trisomics} \)
the minimum number of trisomics required to establish a phenotype shift.

For example, in the haptoglobin system reported in Section 2, it was found that on a sample of 254 Down's subjects the frequency of the $H^p_1$ gene was 0.419 and $H^p_2$ 0.581 such data applied to the top curve in Fig. 2 ($k = 1.5, m = 0.1$) would suggest a minimum number of 1000 Down's to establish a phenotype shift by chi square (see also Table 5). Applying the same data (assuming $N = n = 254$) to the above equations the Ln estimate (assuming haptoglobin was located on chromosome No. 21) would be 0.437 with a variance of 0.13, i.e. with such a gene frequency 254 cases would be sufficient. However when the log ratio test is applied the estimate was found to be 0.164 with a variance of 0.14, i.e. there is no significant phenotype shift.
In this section a critical evaluation of the use of the phenotype shift in the gene location on the trisomic chromosome has been made. It has been shown that when such factors as crossing over, post fertilisation non-disjunction, etc. are taken into account, the use of the simple chi square requires much larger numbers of subjects that were hitherto believed. It was also shown that the use of two gene in the calculations aggravated the problem. Finally it was possible to show that the use of the Haldane log ratio test considerably reduced the numbers required, and this was successfully applied to on the haptoglobin data.
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and Adenylyl Kinase.

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Academic Press.


Chromosomes in Trisomy 21 Down's Syndrome
(Mongolism)

N.Y. Acad. Sci.
"all the business of life is to endeavour to find out what you don't know by what you do, that's what I called guessing what was on the other side of the hill."

Duke of Wellington.
In this final section an attempt will be made to summarise the findings of this investigation and to point out those areas in which further investigations might be profitable.

In the first section, quantitative and qualitative data were presented on a number of serum and plasma proteins and glycoproteins. No evidence of structural changes in the 30 proteins investigated could be detected, nor could a reported change in the $F_c$ fragment of the IgG component be confirmed. The literature on the levels of serum proteins was reviewed and shown to be, for the most part, in conflict. By careful selection of control subjects from among their institutionalised peers, the subjects with Down's syndrome were shown to deviate much less than might be expected after a superficial search of the literature. Contrary to expectation a significant reduction in total protein was noted, principally due to the low levels of albumin encountered, but the contribution of the lower levels of the $\alpha_2$ - and $\beta$ - globulins was seen. A significant reduction in the levels of the $\alpha_1$ - and $\beta$ - glycoproteins was observed, a finding contra-indicated by the observations on the quantitative levels of these proteins by radial diffusion. The divergence of findings by the two techniques was suggested to be
mainly due to technical difficulties with the electrophoretic bands being heterogeneous for a number of proteins for which no anti-sera are available. Two of the four immunoglobulins investigated were found to be elevated (IgA and IgD), and four of the specific proteins appeared suspect when compared with reported normal data ($\alpha_1$-antitrypsin, transferrin, $\alpha_1$-acid glycoprotein and haptoglobin) but comparison with mentally retarded control subjects eliminated all but the $\alpha_1$-acid glycoprotein.

Section 2 dealt with a number of serum and red cell polymorphic systems, and in addition to comparison of the frequency distributions, the data on these were analysed for a number of factors (age, sex, etc.). The most significant finding in this section was of an altered frequency distribution of the Gc system, with an increase in the proportion of the heterozygotes at the expense of both homozygous forms. Reference back to the previous section on the quantitative levels of these proteins was made, and this, together with the reported linkage between the Gc proteins and albumin cast considerable doubt on the location of the Gc system on the trisomic chromosome. In section two a number of phenotype association studies were made with little success, and a number of frequency changes in several systems reported by
other investigators was not upheld. An interesting sex
difference in the phosphoglucomutase system was noted for
the Down's subjects and an apparently abnormal distribution
of haptoglobin frequencies in the control subjects but not
the Down's subjects confirmed a similar finding reported
elsewhere.

Section 3 investigated the effect of parental age on five
polymorphic systems suitable for such an analysis, and a
bimodal distribution in the frequency of the haptoglobin
heterozygotes was observed. This bimodality was in evidence
for both paternal and maternal age, but by analogy with
previous studies it was suggested that the paternal age effect
was the resultant of a high maternal:paternal age correlation.
The possibility that this observation was due to sampling
errors was reduced by the failure to find a similar bimoda-
lity in the other polymorphic systems investigated.

In section 4 the much-reported increased rate of infection in
Down's syndrome was investigated, and a high frequency of
hepatitis-associated antigen (Australia antigen) was en-
countered. Using this antigen as a criteria the Down's sub-
jects were sub-divided into those susceptible to infection and
those not, and nine of the polymorphic systems were compared both singly and in multiples between the two groups. Even after applying a number of statistical techniques no evidence of decreased fitness with respect to any of these polymorphic systems could be detected. In this section also, a number of liver function tests were carried out and comparisons between the antigen positive and antigen negative subjects were made in an attempt to assess if the higher frequency of hepatitis might lead to severe enough liver damage to affect the levels of the serum proteins. The final impression was that if such an effect was in operation the resulting change would be minimal.

Section 5 was confined to a comparison of the Down's group and 54 subjects with Tuberous Sclerosis in an attempt to estimate if selection by mortality played a significant part in either the levels of the individual proteins or in the frequency distributions of the polymorphic systems. In two polymorphic systems (haptoglobin and Gc proteins) abnormal distributions were detected in the Tuberous Sclerosis subjects the suggestion made in Section 3 that the abnormal distribution of Gc protein frequencies in Down's syndrome not being due to the locus being on the trisomic chromosome was strengthened.
Section 7

In Section 6 a critical evaluation of the use of the phenotype shift in the gene location on the trisomic chromosome was made by the development of existing mathematical models to include three alleles at one locus and three alleles one being of the silent variety. Applying these derived equations an estimate was made of the number of Down's subjects required to produce a significant phenotype shift for several gene frequencies and the shortcomings of this and other surveys was pointed out. Using the derived equations it was shown that using pairs of genes had no advantage in reducing the numbers required, but the application of the Haldane log ratio test to the frequency distributions was far superior to the use of the simple chi square.

Future Developments.

The possibilities arising out of the search for new antigenically-identifiable serum proteins and the addition to the range of anti-sera for the estimation of those presently known in serum is sufficiently obvious not to require elaboration, but the investigation of the polymorphic proteins (both genetical polymorphisms and tissue isoenzyme systems) require some brief observations. Most common diseases seem to have a polygenic background, this includes such a diversity as the
common birth defects as well as common diseases of the adult
as the major psychoses, diabetes, peptic ulcer, rheumatic
arthritis, atherosclerosis, hypertension and psoriasis,
where multiple genes appear to interact with unknown factors
to produce the necessary phenotype. Differential population
frequencies are known for birth defects, thus cleft lip and
palate have a high frequency among the Japanese, an inter­
mediate frequency among Europeans and a low frequency among
negro races (1). While no absolute proof exists that these
differences are genetical, this hypothesis is most likely.
What is also likely on a priori grounds is that polymorphisms
affecting developmental proteins exist and will be associated
with small deviations in enzyme activity. With only two
polymorphisms interacting, a given developmental process
could be placed beyond the required threshold and a resulting
developmental defect arise. However a full understanding of
all the genes and all environmental problems affecting a
given process may fail to lead to full understanding or pre­
vention. Identical twins usually have a concordance of less
than 50% for common birth defects while non-identical twins
have concordence rates around 5% (2). Since identical twins
share a common gene pool and a similar uterine environment
this frequent lack of concordence suggests that the processes
may have an indeterminate aetiology. In other words neither hereditary nor environmental factors may adequately account for the defect which is postulated to be caused by random failure of a given developmental sequence to occur.

If we agree with Motulski that the total number of human structural genes to be 50,000 and that some 25% of these are polymorphic then the possibilities would seem endless (3) and taking into account the observations made above the scope would seem infinite. But finite or infinite as the scope may be, this investigation has shown, if nothing else, the need for the careful definition of the Down's subjects under investigation, with factors such as age, sex, maternal age distribution and patterns of infection rate and mortality to be taken into consideration before any valid conclusions can be drawn from the data. The role of the various phenotype distributions in such studies as the susceptibility to disease and mortality, and possibly in the process of non-disjunction will eventually throw more light on the pathogenesis of the syndrome that the simple gene location on the additional chromosome. Finally, for purely logistical reasons two principal lines of investigation remained outside this investigation, namely the foetal proteins in the prospective Down's child, and extensive family phenotyping. It is
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APPENDIX

SELECTION OF SUBJECTS

Down's Syndrome

The 256 Down's subjects which form the basis of this investigation were all resident in two large hospitals for the mentally subnormal, St. Lawrence's Hospital, Caterham (total population 1720) and Royal Earlswood Hospital, Redhill (total population 1145). Both these institutions draw their patients from approximately the same geographical area of South London and Surrey, which is an area of mixed ethnology. By examination of the subjects name, appearance and family background an attempt was made to exclude any who were not of Caucasian origin.

The sample consisted of 114 females and 142 males, the age distribution of the sample is given in Table 1.

The original choice of subject was made on the basis of clinical diagnosis but each case was confirmed by chromosomal analysis of peripheral blood. Only those subjects shown to be trisomic for chromosome 21 were finally accepted, those cases exhibiting mosaicism (4 cases), and translocation
(3 cases) were not included. The chromosomal investigation of only one tissue (in this case peripheral blood) does not totally exclude the possibility of mosaicism in other tissues, and such mosaics might occur in the sample. All the chromosomal investigations were carried out by the Cytogenetical Laboratory at St. Lawrence's Hospital, and I am indebted to the members of that department and to Dr. B.W.R. Richards for making available their records.

Drawing any sample of subjects from an institutionalised population, as this investigation does, is bound to introduce some degree of bias in the sample, and this bias will vary considerably from one institution to another depending on local priorities for admission. In general, admission only takes place when social pressures are present in addition to medical reasons. In a follow-up survey of Down's subjects in Denmark, Oster and van den Tempel (1) showed the reasons for institutionalistic as being:-
### Table 1

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Down's Synd.</th>
<th>Tuberous Scl.</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>Cu.%</td>
</tr>
<tr>
<td>0 - 5</td>
<td>2</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>5 - 9</td>
<td>9</td>
<td>3.5</td>
<td>4.3</td>
</tr>
<tr>
<td>10 - 14</td>
<td>24</td>
<td>9.4</td>
<td>13.7</td>
</tr>
<tr>
<td>15 - 19</td>
<td>35</td>
<td>13.7</td>
<td>27.4</td>
</tr>
<tr>
<td>20 - 24</td>
<td>37</td>
<td>14.5</td>
<td>41.9</td>
</tr>
<tr>
<td>25 - 29</td>
<td>35</td>
<td>13.7</td>
<td>55.6</td>
</tr>
<tr>
<td>30 - 34</td>
<td>40</td>
<td>15.6</td>
<td>71.2</td>
</tr>
<tr>
<td>35 - 39</td>
<td>24</td>
<td>9.4</td>
<td>80.8</td>
</tr>
<tr>
<td>40 - 44</td>
<td>20</td>
<td>7.8</td>
<td>88.4</td>
</tr>
<tr>
<td>45 - 49</td>
<td>10</td>
<td>3.9</td>
<td>92.3</td>
</tr>
<tr>
<td>50 - 54</td>
<td>9</td>
<td>3.5</td>
<td>95.8</td>
</tr>
<tr>
<td>55 - 59</td>
<td>8</td>
<td>3.1</td>
<td>98.9</td>
</tr>
<tr>
<td>60 - 64</td>
<td>3</td>
<td>1.2</td>
<td>100</td>
</tr>
<tr>
<td>65 - 69</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>70 +</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 1.** Age Distribution and Cumulative % Distribution of 256 Subjects with Down's Syndrome, 54 Subjects with Tuberous Sclerosis and 853 Mentally Sub-Normal Control Subjects.

- Mother exhausted.......................... 80
- Broken family............................. 47
- Disturbance of sibs........................ 44
- Physicians advice.......................... 43
- Best for subject.......................... 36
This selection of subjects among those which have proved an unacceptable financial or social burden on their immediate family probably explains the excess of mentally subnormal patients arising from the lower socio-economic groups (2-9). Table 2 compares the social class distribution of the Down's and control groups (as defined in the classification of Occupations 1960 (10)) with the census data for London and the proportional distribution of mentally subnormal subjects (11).

<table>
<thead>
<tr>
<th>Group</th>
<th>Social Class (per thousand)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 + II</td>
</tr>
<tr>
<td>Present survey (Down's)</td>
<td>124</td>
</tr>
<tr>
<td>Present survey (Controls)</td>
<td>149</td>
</tr>
<tr>
<td>Census (London)</td>
<td>170</td>
</tr>
<tr>
<td>Ment. Subnormal Males*</td>
<td>76</td>
</tr>
<tr>
<td>Ment. Subnormal Females</td>
<td>89</td>
</tr>
</tbody>
</table>

* Registrar General's Statistical Review (11).

Table 2. Social Class Distribution per Thousand of the Down's and Control Subjects Compared with Census Data and other Surveys on the Mentally Subnormal.
Appendix

From Table 2 it can be seen that both the Down's and control subjects show a similar excess of subjects of social classes IV and V at the expense of classes I and II, that this bias is less extreme than the data on England and Wales quoted above is probably due to the abnormal socio-economic distribution to be found in S.E. England favouring classes I and II. Whether such a socio-economic bias could influence the data in this investigation has not been pursued as it would require a much greater sample than was available, but in the relevant sections it was noted that there were changes which might arise from such bias (2, 3).

Control Subjects

The control subjects were drawn from one of the two hospitals described above (St. L.) and were not so much selected as those subjects remaining after a number had been rejected for several reasons. A subject was rejected from the sample if there was any case for non-Caucasian origin, or if chromosomal investigation showed any additional or loss of chromosomal material. No subject was included if the records showed any medication for the three months proceeding the investigation (eliminating possible changes through enzyme induction) or if the subject had been exposed to long term medication such as barbiturates which could have led to liver
Appendix

damage. It has been suggested that among the non-specific causes which could bring about biochemical changes (albeit transitory) is the emotional shock of being institutionalised (12) and for this reason no subject was accepted for investigation who had been resident for less than one year.

After selection as outlined above, a control pool of 853 subjects remained from which, at random the various control groups described in the various sections were drawn. The age distribution of these 853 (198 females and 655 males) is shown in Table 1 above. For purely logistic reasons, not all of these control pool could be investigated for each parameter, but there is no reason to suppose that the smaller control groups differ in either age distribution or kind from the larger control pool. The control pool and the Down's group being drawn from the same hospital populations could be assumed to be in receipt of similar diets and vaccination regimes. In neither the Down's nor the control pool were any subjects in receipt of specialised diets.

Any attempt to list the diagnoses of the control pool would create the false impression that the clinical cause of their subnormality was known. In any mentally subnormal population
the greater majority of cases can only be classified in terms of the severity of the reduced intelligence, or in terms of the approximate dating of the initial insult (prematal, perinatal or post-natal etc.), classifications which are crude even at the best. In his survey of 800 cases admitted in a ten year period at the Fountain Hospital, Berg (13) could only establish specific aetiological factors or distinct syndromes for only one third of the cases. Table 3 below divides the control pool into three degrees of severity of mental defect, idiot (i.e. IQ 0 - 50), Imbecile (IQ 51 - 80) and Feebleminded (IQ 81 - 100) by age.
### Table 3

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Idiot</th>
<th>Imbecile</th>
<th>Feebleminded</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 - 9</td>
<td>1</td>
<td>19</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>10 - 14</td>
<td>2</td>
<td>32</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>15 - 19</td>
<td>4</td>
<td>54</td>
<td>10</td>
<td>68</td>
</tr>
<tr>
<td>20 - 24</td>
<td>5</td>
<td>58</td>
<td>11</td>
<td>74</td>
</tr>
<tr>
<td>25 - 29</td>
<td>5</td>
<td>66</td>
<td>13</td>
<td>84</td>
</tr>
<tr>
<td>30 - 34</td>
<td>6</td>
<td>76</td>
<td>14</td>
<td>96</td>
</tr>
<tr>
<td>35 - 39</td>
<td>6</td>
<td>75</td>
<td>14</td>
<td>95</td>
</tr>
<tr>
<td>40 - 44</td>
<td>5</td>
<td>74</td>
<td>13</td>
<td>92</td>
</tr>
<tr>
<td>45 - 49</td>
<td>4</td>
<td>50</td>
<td>9</td>
<td>63</td>
</tr>
<tr>
<td>50 - 54</td>
<td>3</td>
<td>41</td>
<td>8</td>
<td>52</td>
</tr>
<tr>
<td>55 - 59</td>
<td>3</td>
<td>41</td>
<td>8</td>
<td>52</td>
</tr>
<tr>
<td>60 - 64</td>
<td>2</td>
<td>41</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>65 - 69</td>
<td>2</td>
<td>29</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>70 +</td>
<td>1</td>
<td>22</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>678</td>
<td>126</td>
<td>853</td>
</tr>
</tbody>
</table>

Table 3. 853 Mentally Subnormal Subjects in the Control Pool in Three Degrees of Severity of Mental Defect by Age (Years).
Appendix

Tuberous Sclerosis

Section 5 reports on the comparison of 54 subjects with Tuberous Sclerosis (T.S.) and the Down's subjects and control groups in an attempt to estimate any effect on either circulating protein levels or phenotype distributions by selection by mortality. In the selection of the subjects with T.S., the mortality data of Down's subjects as reported in the literature was considered and compared with the reported mortality figures for T.S. This data is compared in Fig. 1, the Down's data is based on a number of surveys (14 - 19), and it will be seen that in the 0 - 5 year age group two curves are represented, the upper curve is based on the earlier data of Carter (14) and Record & Smith (19) and predating the general use of antibiotics show a significantly higher mortality.

![Mortality Data graph](image)

**Fig. 1.** Mortality Data (from published data) on Down's Syndrome (O - 0 pre 1950, X - x post 1950) and T.S. (O - O).
Mortality data on T.S. is much more scanty and less reliable than that on Down's syndrome. Bielchowsky and Gallus (20) in a survey on 78 cases reported death in 22 cases by the 5th year, 35 by the 10th year, 67 by the 25th year and 76 by the 50th year. Vaas on 126 cases reported a mere 3% death by the end of the first year, 28% by the 10th year and 75% by the 25th year (21). Shrie & Bornstein (22) reported 30% dead by the 5th year and 75% by the 20th year. These data are combined and smoothed by eye in Fig. 1.

The cumulative mortality of 30% for T.S. subjects of 5 years or less is probably a gross under-estimate. The majority of the cases are originally diagnosed by the triad of features, mental subnormality, epilepsy and sebaceous adenoma with only the latter feature differentiating the syndrome from a number of other causes of epilepsy and mental subnormality. The appearance of the sebaceous adenoma, however, is often late, Dawson (23) reported the appearance between 5 and 13 years, in their report on eight cases De La Cruz and La Veck (24) gave a mean of 4.5 years and Kofman & Hyland (25) reported that the appearance of the sebaceous adenoma could be as late as 26 years. This late appearance of the principal diagnostic feature would bring about a number of missed cases in the
0 - 5 year group which would increase the mortality probably to the 50\% mark of the Down's subjects. In this respect it is of interest to note that in his survey on a number of families Borberg (26) found only one case of T.S. in each of the 0 - 5 and 5 - 10 groups both of whom were dead, and of four cases in the 10 - 15 year group three were dead.

Taking the above data on the mortality in the Down's and T.S. group it seems reasonable to suggest that both show very similar mortality rates in the first five years after birth with some half of them not reaching their fifth birthday, and if selection by mortality was in operation then the maximum pressure would be exerted during this period. Of course it is possible that this selection in the first five years of life is just a continuation of factors operating in prenatal periods, but unfortunately there is little reliable evidence of prenatal death rates in Down's syndrome and none at all for T.S.

The T.S. subjects investigated in Section 5 were all resident in a number of hospitals for the mentally subnormal, and all presented the triad of diagnosable features of mental subnormality, epilepsy and sebaceous adenoma. All the subjects were selected by age, only those over 10 years were included
and the age distribution of the 54 cases investigated are shown in Table 1.
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