HYDROLYTIC CLETOLYTIC ENZYMES INVOLVED IN GLYCOCEN METABOLISM

A Thesis submitted by
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Doctor of Philosophy
in
BIOCHEMISTRY

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January 1972
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere thanks to Professor J.B. Pridham for his guidance and encouragement during the supervision of this work. I wish to thank Professor E.J. Bourne for the interest taken in this work and for his helpful discussions. I also wish to thank Dr. P. Golding of the London Hospital for supplying a human liver biopsy specimen. I am indebted to the Medical Research Council for financial support.

I would like to give special thanks to my mother and father and to my wife and children, without whose patience and understanding this Thesis would not have been possible.

To purify rat liver lysosomes by rate-zonal centrifugation through a sucrose-density gradient. The purity of the organelle prepared by this method has been studied by biochemical analyses and electron microscopy.

The action of various hormones on rat liver α-glucosidases has been examined. Cortisone acetate was observed to activate rat liver acid α-glucosidase, but not the neutral enzyme, in vivo, liver slices and purified lysosomes and in human liver tissue. It was noted that for activation of the enzyme in lysosomes to occur, intact organelles were necessary; when 14C-4-cortisone acetate was incubated with purified lysosomes, 76% of the total activity recovered in the organelles was present in the minute fraction and this suggested that the cortisone played an important role in the activation phenomenon. In addition, it was only the soluble enzyme, and not the membrane-bound enzyme, that was activated. Changes in the structure of the steroid resulted in a loss of the activation property. Some other lysosomal hydrolases were not affected by cortisone acetate.
Acid and neutral $\alpha$-glucosidases, with apparent molecular weights of 179,900 and 27,500, respectively, have been partially purified from rat liver tissue. The neutral enzyme is contaminated with an acid $\alpha$-glucosidase of similar molecular weight. The high molecular weight acid $\alpha$-glucosidase can be dissociated into inactive sub-units by 8M-urea.

Liver fractionation studies confirm that the high molecular weight acid $\alpha$-glucosidase is located in the lysosomal fraction, in soluble and membrane-bound forms, whereas the neutral enzyme (with its associated acid enzyme) is present in the microsomal fraction.

A large-scale preparative technique has been devised to purify rat liver lysosomes by rate-zonal centrifugation through a sucrose-density gradient. The purity of the organelles prepared by this method have been studied by biochemical analyses and electron microscopy.

The action of various hormones on rat liver $\alpha$-glucosidases has been examined. Cortisone acetate was observed to activate rat liver acid $\alpha$-glucosidase (but not the neutral enzyme) in vivo, liver slices and purified lysosomes and in human liver tissue. It was noted that for activation of the enzyme in lysosomes to occur, intact organelles were necessary. When $^{14}$C-4-cortisone acetate was incubated with purified lysosomes, 78% of the total activity recovered in the organelles was present in the membrane fraction and thus suggested that the membrane played an important role in the activation phenomenon. In addition, it was only the soluble enzyme, and not the membrane-bound enzyme, that was activated. Changes in the structure of the steroid resulted in a loss of the activation property. Some other lysosomal hydrolases were not affected by cortisone acetate.
**Kₘ and Vₘₐₓ determinations** suggested that cortisone acetate acted by increasing the catalytic efficiency of the lysosomal acid α-glucosidase. However, the intralysosomal glycogen level in vivo was not lowered when the rats were treated with cortisone acetate.

Possible mechanisms for the enzyme activation are discussed.

**Abbreviations**

**Introduction**

1. Structure of Glycogen
2. Enzymes involved in Glycogen Synthesis
3. Enzymes involved in Glycogen Degradation
4. Hormonal Control of Glycogen Metabolism
5. Glycogen Storage Diseases
6. Lysosomes

**Results and Discussion**

1. Purification and Properties of Rat Liver α-glucosidases
2. Hormonal Control of Acid α-glucosidase

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<tr>
<td>Acetyl CoA</td>
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<td>AMP</td>
<td>Adenosine 5'-phosphate</td>
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<td>Adenosine 5'-diphosphate</td>
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<td>Adenosine 5'-triphosphate</td>
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<tr>
<td>CTP</td>
<td>Cytosine 5'-triphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>Man-6-P</td>
<td>D-Mannose-6-phosphate</td>
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<tr>
<td>PEP</td>
<td>Phosphoenol pyruvate</td>
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<tr>
<td>POPOP</td>
<td>1,4-Di-[2-(5-phenyloxazolyl)]-benzene</td>
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<tr>
<td>PPO</td>
<td>2,5-Diphenyloxazole</td>
</tr>
<tr>
<td>UMP</td>
<td>Uridine 5'-phosphate</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine 5'-diphosphate</td>
</tr>
<tr>
<td>UTP</td>
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<td>Uridine 5'-diphosphoglucone</td>
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<tr>
<td>UDPGal</td>
<td>Uridine 5'-diphosphogalactose</td>
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The Structure of Glycogen

Glycogen is the reserve polysaccharide of animal tissues which serves as a store of carbohydrate. For maintenance of the blood-sugar level and, indirectly, as a source of energy for muscular activity and for various metabolic processes, particularly during synthesis and growth.

Glycogen has been isolated from liver, brain, skeletal and cardiac muscles, placenta, skin, intestinal mucosa and adipose tissue (1). Glycogen is also present in the tissues of invertebrates and in micro-organisms (2).

Glycogen consists primarily of chains of 2-glucose units joined by α-1,4-glycosidic linkages. In addition, 6-10% of the linkages are α-1,6 and these give rise to a branched structure. This has been shown by methylation studies (2), degradative enzymic techniques (3), periodate oxidations (4) and fragmentation analysis (5).

The yields of 2,3,4-tri- and 2,3,6-tri and 2,3,4,6-tetra-O-methylglucoses from hydrolysed methylated glycogen led to an early suggestion (5) that glycogen is a linear polymer of α-1,4-glucose units long joined to each other at α-1,6-branch points (2).

**INTRODUCTION**

The structure (1) consists of α-1,4-β-linked glucose units long joined to each other at α-1,6-branch points (2).

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**“LAMINATED” STRUCTURE**

- α-1,4-linkages
- α-1,6-linkages
- branch point
- terminal end unit

Stroudinger and Hasegawa (6), who also carried out methylation
The Structure of Glycogen

Glycogen is the reserve polysaccharide of animal tissues which serves as a store of carbohydrate for maintenance of the blood-sugar level and, indirectly, as a source of energy for muscular activity and for various metabolic processes, particularly protein synthesis and growth.

In mammals glycogen has been isolated from liver, brain, skeletal and cardiac muscles, placenta, skin, intestinal mucosa and adipose tissue (1). Glycogen is also present in the tissues of invertebrates and in micro-organisms (1).

Glycogen consists primarily of chains of D-glucose units joined by α-1,4-glycosidic linkages. In addition, 6-10% of the linkages are α-1,6 and these give rise to a branched structure. This has been shown by methylation studies (2), degradative enzymic techniques (3), periodate oxidations (4) and fragmentation analysis (5).

The yields of 2,3-di- and 2,3,6-tri- and 2,3,4,6-tetra-O-methylglucoses from hydrolysed methylated glycogen led to an early suggestion that glycogen had a "laminated" structure (1) consisting of α-1,4-linked D-glucose chains, 10-18 units long joined to each other at α-1,6-branch points (2).

"LAMINATED" STRUCTURE

Staudinger and Husemann (6), who also carried out methylation
studies, suggested the "comb" structure (II) for the polysaccharide and proposed that it was built up of a long chain of up to 100 α-1,4-linked D-glucose units to which were attached short side chains of 12-19 D-glucose residues joined at the C-2, C-3 or C-6 positions of the main chain units.

The technique of β-amylolysis was also used to provide further information regarding the structure of glycogen. Meyer and Fuld (7) incubated a sample of mussel glycogen with β-amylase and found that 47% of the glycogen was converted to maltose. Methylation analysis showed that the chain length of the glycogen was 11 D-glucose units, whilst that of the β-amylase limit dextrin was 5.5 units. Meyer then introduced the concept that glycogen had a highly branched, tree-type structure (III) with exterior chains containing 6-7 D-glucose units and interior chains with 3 D-glucose units.
MEYER "TREE" STRUCTURE

A linear chains of α-1,4-linked D-glucose units.

- α-1,6-interchain linkages

The chains constituting the glycogen molecule have been termed A, B, and C (8). The arrangement of these chains is shown in the diagram. It is probable that only one free reducing glucose residue, R, is present in the molecule and that this terminates the single C-chain. Diagrammatic representations of this structure usually show the A-chains on the periphery of the molecule. However, French has raised the possibility that a small number of A-chains may be "buried" within the interior of the molecule (9). Such chains would be less accessible to enzymic degradation, as due to incomplete oxidation.

Meyer and Bernfeld (10) provided additional evidence for the multibranched structure of glycogen by carrying out a stepwise enzymic degradation using β-amylase and a yeast enzyme preparation containing "isomaltase". Incubation of glycogen with
β-amylase yielded a limit dextrin. Incubation of this dextrin with the 'isomaltase' rendered it susceptible to further degradation by the β-amylase to give a second limit dextrin.

Conclusive experimental proof of the multibranched structure of glycogen was established by the alternate use of a debranching enzyme (amylo-1,6-glucosidase) and muscle phosphorylase (11). It was shown that successive tiers of side chain branches could be removed from the glycogen molecule by this process.

Various chemical methods, in addition to methylation, have been used to obtain evidence for the branched structure of glycogen. For example, glycogens were oxidised by potassium metaperiodate by Halsall, Hirst and Jones (4) and the resulting yields of formic acid used to calculate the average chain lengths of the molecules. The results were in agreement with those determined by methylation. This technique provided an additional method for an estimation of the glycosidic linkages in the molecule (12); 97-99% of the interchain linkages shown to be α-1,6.

Abdel-Akher et al (13) extended this technique by reducing the periodate oxidised glycogen before hydrolysis (Smith degradation). A quantitative examination of the resulting alcohols gave data regarding chain lengths and the nature of the glycosidic bonds in the polysaccharide. Hence, the ratio of glycerol to erythritol was found to be 1:10, that is, indicating an average chain length of 11 D-glucose units, which is in good agreement with values obtained by methylation analysis (13). Gibbons and Boissannas (14) found glucose to be present after periodate oxidation of glycogen and suggested that glycogen contained some 1,3- or 1,2-linkages. However, Bell and Manners (12) could not substantiate this claim and suggested that the presence of glucose was due to incomplete oxidation.

Partial acid hydrolysis has been used in the elucidation of many polysaccharide structures. The isolation of isomaltose (5) (15), panose, maltotriose and maltotetraose (16) containing α-1,4, α-1,6
and mixed α-1,4-, 1,6 linkages from a partial acid hydrolysate confirmed the nature of the glycosidic linkages in the main chains and at the branch points in glycogen.

In later investigations, Wolfrom and Thompson (17) isolated isomaltotriose and nigerose from the hydrolysates of liver glycogen which they said arose from adjacent α-1,6- linkages in the molecule, and small numbers of α-1,3- linkages respectively. The chemical evidence for this is not believed to be conclusive (18) owing to the possibility of transglucosylation reactions occurring.

Infrared spectroscopic analysis has provided information concerning the nature of the linkages and the stereochemistry of the anomeric carbon atoms in the intact glycogen molecule. Three characteristic absorption peaks were observed when the polysaccharide was examined over the frequency range of 730-960 cm⁻¹ (19). The peak at 835 cm⁻¹ is characteristic of carbohydrate polymers containing α-glucosidic linkages, while those at 762 and 928 cm⁻¹ result from α-1,4- linked D-glucose units.

Studies on the action of salivary α-amylase on glycogen have provided qualitative evidence of multiple-branching (20). The products of α-amylolysis included maltose, maltotriose and a series of α-limit dextrans containing α-1,6- linkages. Incubation of the α-limit dextrans with R-enzyme (which hydrolyses the outermost α-1,6-interchain linkages) resulted in the cleavage of α-1,6- linkages and the liberation of reducing sugars. Measurement of the amount of sugar produced enabled the percentage α-1,6- linkages and hence, the average chain length to be calculated (21). However, at high concentrations of α-amylase, maltotriose and dextrans were further degraded with the liberation of glucose (22). Under these conditions, there is a relationship between the amount of reducing sugar produced and the average chain length (CL) of glycogen (23):
P_{1,6} = \frac{100}{\text{CL}} = 23.3 - 0.21 (P_{M})_{1,6} \%

P_{1,6} \% 1,6\text{-} linkages

\overline{\text{CL}} \text{ chain length } (\text{CL})

P_{M} \% \text{ reducing sugar}

The average interior (\overline{\text{ICL}}) and average exterior (\overline{\text{ECL}}) chain lengths have also been determined by a similar method using \( \beta \)-amylolysis (24). Chain lengths can also be found by means of an iodine staining method (25). This is dependent on an observed relationship between the wave length of maximum absorption of the glycogen-iodine complex (in half saturated ammonium sulphate solution) and chain length (CL):

\overline{\text{CL}} = 16 + 0.114 (\lambda_{\text{max}} - 500)

French (26) has investigated \( \alpha \)-limit dextrins and showed that some contain two \( \alpha \)-1,6\text{-} branch points separated by three glucose units or less. This suggested areas of multiple branching. Other evidence of multiple branching has been obtained by the calculation of the A:B-chain ratio of various glycogens (27).

An examination of the structure of shell-fish glycogen using isoamylase from \textit{Cytophaga} has recently been carried out by Gunja-Smith et al (28). This enzyme hydrolyses the branch linkages of glycogen and leads to complete debranching of the polysaccharide; it will not remove 1,6\text{-}linked \( \alpha \)-maltosyl residues from a polysaccharide. Glycogen was degraded successively with muscle phosphorylase and \( \beta \)-amylase to yield a glycogen \( \varnothing \), \( \beta \)-dextrin in which A-chains are reduced to two glucose units (29). Incubation of the dextrin with isoamylase resulted in the hydrolysis of B\( \rightarrow \)B linkages (see structure IV over page), but only half the number of branch points were hydrolysed that were hydrolysed by a mixture of isoamylase and pullanase. The latter enzyme will remove 1,6\text{-}linked \( \alpha \)-maltosyl stubs from dextrins (30). The degree of
β-amylolysis of isoamylase-treated α, β-dextrin was found to be 44% and not nil. This suggested that either, many B-chains do not carry A-chains, or, if they do, the A-chains are not available to phosphorylase and β-amylase. Gunja-Smith et al (29) proposed a model (IV) to explain these results, taking into account the “buried” chains concept of French (9):

In summary, therefore, the established model for glycogen structure is that of a polymerscope, high molecular weight polymer of α-glucose. The glucose units are primarily linked together by α-1,4- bonds, but α-1,6-bonds are also present in smaller amounts. The glycogen molecules are very highly branched and it has been calculated that steric factors would limit the molecular weight to 4-20 x 10⁶, depending on the degree of hydration. The calculation, due to French (9), assumed a volume of 2000A³ for each glucose unit.
based on X-ray measurements, and an average chain length of 12 units. However, the molecular weight of glycogen seems to be governed by the method of extraction (31). Glycogen, can be extracted using hot (32) or cold (33) water, hot alkali (34) (35) or trichloroacetic acid solution (36) (37). Molecular weight determinations have been made by osmotic pressure (38), light scattering (37) and sedimentation methods (39). Hot water and alkali-extracted glycogens were observed to have molecular weight values ranging from $2.5 \times 10^6$ (32) (38), whereas glycogens obtained by trichloroacetic acid extractions had higher molecular weights in the region of $11-80 \times 10^6$ (37). It has been claimed that glycogen extracted with dimethyl sulphoxide more closely resembles "native" glycogen than preparations obtained by other methods of extraction; higher molecular weight preparations are obtained with dimethyl sulphoxide (39). Bell et al isolated glycogen containing two polydisperse components with sedimentation coefficients of 60-100S and 150-300S (40). Cold water extraction of glycogen from Ascaris muscle has produced two fractions of molecular weight $450 \times 10^6$ and $50 \times 10^6$, but extraction by other methods degraded the polysaccharide (31).

In summary, therefore, the established model for glycogen structure is that of a polydisperse, high molecular weight polymer of D-glucose. The glucose units are primarily joined together by $\alpha$-1,4-linkages into chains that on average, vary from 10 to 18 units in length, but also at the points in the molecule where individual chains connect, by $\alpha$-1,6-linkages, to form a multiply-branched, polymolecular structure. Investigations have been carried out on the state of glycogen in the tissues, and it has been observed to occur in two forms. One form is extractable with water or trichloroacetic acid solution under mild conditions, while the other is extracted by more drastic means such as with hot alkali (41). The former has been classified as "lyoglycogen" (free) and the latter "desmoglycogen" (residual) (42). Roe et al (43) stated that the concept of lyo- and desmo- glycogen was false and that
it was due to incomplete tissue homogenisation. These differences disappeared when glycogen was extracted from tissues by homogenisation in cold trichloracetic acid solution with glass beads. It has been suggested that the residual glycogen is possibly bound to protein (44) and that this protein-glycogen link can be cleaved by proteinases (44). However, it is known that glycogen is associated with various enzymes, including phosphorylase (45) (46), glycogen synthetase and branching enzyme (47). A rabbit muscle protein-glycogen complex has been obtained and separated into light (120S) and heavy (600S) fractions (48). The light fraction consisted mainly of glycogen particles with associated phosphorylase, phosphorylase kinase and phosphatase, whereas the heavy fraction contained mainly the elements of the sarcoplasmic reticulum.

Electron microscopy of liver glycogen has shown a compound macromolecular structure in which three levels of organization can be recognised (49). Glycogen appeared in clusters or rosettes (α-particles) of 60-200 μm diameter, which were freely dispersed in the cytoplasm (50) (51), and these were composed of smaller round or slightly polyhedral units 20-40 μm in diameter (β-particles). The β-particles consisted of a regular arrangement of smaller, rod-like elements, 3 x 20 μm (γ-particles). The β-particles can be subdivided further into β₁₋, β₂₋, β₃₋ particles, differing in size and molecular weight; this appears to be dependent on the method of breakdown of the α-particles (52). However, in muscle, the glycogen exists as β-particles (15-40 μm diameter) and no rosettes occur. It has been shown that when glycogen is stored in muscle or in liver tissue, the individual particles increase in size without increasing in number (47) (53).

Other interesting investigations have shown an ultrastructural association of intracytoplasmic smooth membranes and glycogen particles, termed the glycogen body (54). It is thought that the enzymes necessary for the synthesis, lysis and mobilization of glycogen are
formed in the rough-surfaced membranes and then transferred to the smooth membranes. The smooth membranes could also initiate the synthesis of glycogen by facilitating the steric arrangement of glucose molecules (55). It has been suggested that ribosomes play some specific role in the formation of new glycogen particles (56) since this formation is accompanied by the transition of rough membranes to smooth membranes.

To start with simple monosaccharides such as glucose and galactose which are converted by a series of reactions into the complex polysaccharide. This overall process is one which results in storage of energy and this store is available for anaerobic reactions and transport and mechanical processes. In this present section the enzymes of glycogen synthesis and breakdown will be discussed separately.

The postulated pathways for glycogen metabolism are diagrammatically represented in Fig.1.

A. Glycogen Synthesis.

The initial reaction in the conversion of glucose to glycogen is phosphorylation of the hemose at C-6 which is catalyzed by the enzyme hexokinase.

Hexokinase (ATP:hexose 6-phosphotransferase; EC 2.7.1.1): In the presence of ATP, hexokinase converts a number of structurally related hexoses to the corresponding hexose-6-phosphates, probably by a direct transfer of the phosphoryl group from the donor to the acceptor without the involvement of phosphohexose-enzyme intermediates (57). The enzyme requires Mg$^{2+}$ for activity (58) and animal hexokinases are inhibited by 6-6-P and hence there would appear to be a feedback mechanism, in situ, (59) although its physiological significance is not clear.

In rat tissues, three hexokinases have been found (Types I, II and III) and these differ particularly with respect to the effect of glucose concentration on the rate of phosphorylation (50). The three forms have been found to be a general characteristic of mammalian species (61).
**Enzymes of Glycogen Metabolism**

The majority of the chemical reactions that occur in the living cell are catalysed by enzymes. Anabolism, where a sequence of reactions leads to a net synthesis of organic materials, and catabolism, where the enzyme-catalysed reactions are primarily degradative, together constitute metabolism. Glycogen metabolism can be considered to start with simple monosaccharides such as glucose and galactose which are converted by a series of reactions into the complex polysaccharide. This overall process is one which results in storage of energy and this store is available for anabolic reactions and transport and mechanical processes. In this present section the enzymes of glycogen synthesis and breakdown will be discussed separately.

The postulated pathways for glycogen metabolism are diagrammatically represented in Fig. 1.

A. **Glycogen Synthesis.**

The initial reaction in the conversion of glucose to glycogen is phosphorylation of the hexose at C-6 which is mediated by the enzyme hexokinase.

**Hexokinase (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1):** In the presence of ATP, hexokinase converts a number of structurally related hexoses to the corresponding hexose-6-phosphates, probably by a direct transfer of the phosphoryl group from the donor to the acceptor without the involvement of phosphoryl-enzyme intermediates (57). The enzyme requires Mg$^{2+}$ for activity (58) and animal hexokinases are inhibited by G-6-P and hence there would appear to be a feedback mechanism, **in situ**, (59) although its physiological significance is not clear.

In rat tissues, three hexokinases have been found (Types I, II and III) and these differ particularly with respect to the effect of glucose concentration on the rate of phosphorylation (60). The three forms have been found to be a general characteristic of mammalian species (61).
Fig. 1. Pathways of glycogen metabolism

Enzymes:

1. α-Amylase (EC 3.2.1.1)
2. α-Glucosidases (EC 3.2.1.20)
3. Glycogen synthetase (EC 2.4.1.11) and branching enzyme (EC 2.4.1.18)
4. Glycogen phosphorylase (EC 2.4.1.1) and amylol-1,6-glucosidase (EC 3.2.1.20)
5. UDP glucose 4-epimerase (EC 5.1.3.2)
6. UDP galactose pyrophosphorylase (EC 2.7.7.10)
7. Galactokinase (EC 2.7.1.1)
8. UTP pyrophosphorylase (EC 2.7.7.9)
9. Mannose-1-phosphate uridyltransferase (EC 2.7.7.12)
10. Hexokinase (EC 2.7.1.1) and glucokinase (EC 2.7.1.2)
11. Glucose-6-phosphatase (EC 3.1.3.9)
12. Phosphoglucomutase (EC 2.7.1.1)
13. Glucosephosphate isomerase (EC 5.3.1.9)
14. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
15. Mannosephosphate isomerase (EC 5.3.1.8)
16. NADP-glucose dehydrogenase (EC 1.1.1.4)
17. Phospho-fructokinase (EC 2.7.1.1)
18. Transaldolase (EC 2.2.1.2) and transketolase (EC 2.2.1.1)
19. Gluconokinase (EC 2.7.1.13)
20. Phosphoglucomutase dehydrogenase (EC 1.1.1.44)
21. Fructokinase (EC 2.7.1.4)
22. Ketohexokinase (EC 2.7.1.1)
Differential centrifugation methods showed that hexokinase was located on the outer membrane of mitochondria (62) and it has been suggested that the physiological significance of this binding may account for the hormonal control of hexokinase activity. Kosow and Rose (63) found that the free enzyme was more sensitive to inhibition by G-6-P than the mitochondrial enzyme.

Glucokinase (ATP:D-glucose 6-phosphotransferase; EC 2.7.1.2): This enzyme is specific for glucose and is the main glucose-phosphorylating enzyme in the liver and it appears to be restricted to this tissue (64). Cellular distribution studies showed that glucokinase was present in hepatocytes, whereas hexokinase was mostly present in non-parenchymal tissues (65). Parry and Walker (66) purified glucokinase from rat liver and showed that the enzyme was also inhibited by glucose-6-phosphate.

An interesting finding is that glucokinase appears in rat liver only some time after birth and its presence in the adult animal depends on induction by insulin (67). Hexokinase, however, is present in fetal liver (68).

Phosphoglucomutase (α-D-Glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase; EC 2.7.5.1): G-6-P lies at the 'crossroads' of glucose metabolism. It may follow a synthetic pathway and be incorporated into glycogen or it may be degraded by glycolysis. G-6-P may also leave the hexose monophosphate pool via the pentose phosphate pathway. In glycogen synthesis G-6-P is isomerized to G-1-P by phosphoglucomutase. Najjar (69) has represented this interconversion by the following scheme:-

\[
\begin{align*}
G-1-P + \text{Phosphoenzyme} & \quad \xrightarrow{\text{Phosphoglucomutase}} \quad G-1,6-diP+\text{Dephosoenzyme} \\
G-1,6-diP+\text{Dephosoenzyme} & \quad \xrightarrow{\text{Phosphoglucomutase}} \quad G-6-P + \text{Phosphoenzyme}
\end{align*}
\]
The process consists of the reversible transfer of either of the phosphate groups of G-1,6-diP to a site on the dephosphoenzyme. This site was shown to be one of the enzyme's many serine residues (70). Confirmation of phosphate transfer involving phosphorylation of the phosphoglucomutase has been obtained using $^{14}\text{C}$ and $^{32}\text{P}$ labelled substrates (71).

Mg$^{2+}$ ions have been shown to be necessary for enzyme activity. A complex appears to be formed with a Mg$^{2+}$-enzyme ratio of 1:1. The metal ion plays a part at the catalytic site of the reaction and also modifies enzyme activity by changing the protein structure (72)(73).

UDPG pyrophosphorylase (UTP:α-D-glucose 1-phosphate uridyltransferase; EC 2.7.7.9): The connecting link between the anabolic and the major catabolic pathway of glycogen is UDPG pyrophosphorylase which catalyses the interconversion of G-1-P and UDPG.

This enzyme has been crystallised from calf liver (74) and constitutes 0.3% of the extractable liver protein. It is Mg$^{2+}$-dependent and catalyses the following reaction:

$$G-1-P + UTP \rightleftharpoons UDPG + PP_i$$

The reaction is freely reversible but UDPG pyrophosphorylase favours UDPG formation (equilibrium constant of 0.28 - 0.34 at pH 7.8 in the direction of UDPG formation) (74).

Inorganic phosphate and UDP have been shown to inhibit the enzyme and this indicates that excess of P$_i$ within the cell would suppress UDPG formation and hence glycogen synthesis (75). UDP, a product of glycogen synthesis from UDPG, might well accumulate in the tissues in the absence of ATP and this would constitute a further control of glycogen synthesis.

The cell fractionation studies of Maddaiah and Madsen (76) have shown that UDPG pyrophosphorylase is located in the microsomal fraction of rat liver. These workers suggested that this microsomal fraction could have a functional significance in that it also carries glycogen synthetase and glycogen phosphorylase.
Glycogen Synthetase (UDP glucose:glycogen α-4-glucosyltransferase; EC 2.4.1.11): Glycogen synthetase has been isolated from skeletal muscle and liver in two forms (77); these were termed I and D. The D-form was found to be dependent for its activity on the presence of G-6-P whereas the I-form was independently active (78). De Wulf et al (79) obtained similar results with the mouse liver enzyme but these workers used a different terminology and referred to synthetases I and D as α and β, respectively. It was suggested that the D-form remained inactive in vivo due to the effect of intracellular $P_i$ and ATP, whereas the I-form remained active in vivo because ATP inhibition was offset by physiological concentrations of $P_i$. It appeared that in the cell, $P_i$ was the most important activator of the I-form of the enzyme and that G-6-P had a minor role (79). Similar conclusions have been reached by other workers (80) (81). Blatt et al (82) suggest that an increase in the G-6-P level could relieve the ATP inhibition of glycogen synthetase to a certain degree and thus make the enzyme more functional.

As glycogen synthesis proceeds, an increase in the level of UDP occurs which inhibits liver synthetase in competition with activation by G-6-P (83). Thus, when glycogen synthesis takes place and UDP accumulates, self regulation would occur in that synthetase, would be inhibited and the falling level of UDPG would permit increased phosphorylation.

Two forms of synthetase I; one 'glycogen-induced', not inhibited by UDPG, and another 'normal' inhibited by UDPG, are reported to occur in bovine heart preparations (84).

Friedman and Larner (85) showed that when partially purified muscle synthetase preparations were incubated with $^{32}$P-ATP and Mg ions, the I-form was converted to a phosphorylated D-form.

$$\text{Synthetase I} + \text{ATP} + \text{Mg}^{2+} \xrightarrow{\text{Kinase}} \text{synthetase D} + \text{ADP}$$
The protein factor which catalyses this reaction, i.e., glycogen synthetase I kinase (ATP: protein phosphotransferase; EC 2.7.1.37), has been separated from glycogen synthetase (86). Both muscle (87) and liver (79) (81) kinases are stimulated by cyclic-3',5'-AMP and other cyclic nucleotides, for example, cyclic-3',5'-UMP. It is not clear whether these other nucleotides have a regulatory function in vivo (80) although such compounds are present in rat brain at concentrations similar to those of cyclic-3',5'-AMP.

Alternative mechanisms for the conversion of I to D have been reported. One is Ca²⁺-dependent and requires a heat-labile, protein factor, the calcium activating factor, (89) and the other involves trypsin (90). The Ca²⁺-dependent reaction is not stimulated by ATP, Mg²⁺ or cyclic-3',5'-AMP and is irreversible (89). It was suggested that the Ca²⁺-dependent conversion of I to D accompanied muscle contraction.

The D to I conversion was observed to occur spontaneously in fresh, crude enzyme extracts with the release of P_i from the protein, which indicated that the reaction was catalysed by a phosphatase (81).
The synthetic action of glycogen synthetase has been summarised as follows (91):

\[
\text{UDPG + (G)_n} \xrightarrow{\text{Glycogen synthetase}} \text{UDP + (G)_{n+1}}
\]

Goldemberg (92) stated that for maximum rates of synthesis glycogen synthetase required a branched macromolecule as a primer. The rate of synthesis of \(\alpha-1,4\) bonds by glycogen synthetase decreased as the outer chains of the glycogen primer molecule were extended (93). Branching Enzyme (\(\alpha-1,4\)-glucan: \(\alpha-1,4\)-glucan 6-glycosyltransferase; EC 2.4.1.18): Enzymes which form \(\alpha-1,6\) branch points in glycogen have been isolated from skeletal muscle (94,95), liver (96), Arthrobacter globiformis, and Escherichia coli (97). Larner (94,98) showed that liver branching enzyme introduced further branching into already branched molecules that had an average outer chain length of between 6 and 11 glucose residues. Verhue and Hers (99) incubated liver branching enzymes with glycogen containing radioactive non-reducing terminal groups. Periodate oxidation of the product showed that the \(^{14}C\)-glucose remained in a peripheral position after branching and pullulanase yielded radioactive malto saccharides, the smallest being maltoheptaose. It was concluded that the enzyme preferentially transferred intact chains of 7 glucose units from a \((1\rightarrow4)\) to a \((1\rightarrow6)\) position and did not function by sequential transfer of single glucose units. Similar conclusions have been reached with rabbit muscle branching enzyme (100) in which maltoheptaose units were preferentially transferred.

There is no evidence to establish whether the transfer of maltoheptaose units is an intra-chain or an inter-chain process. Both types of transfer reaction are shown in Fig.2 (75). Brown et al (101) suggested that the rate of chain lengthening would be greatest if the maltoheptaose unit underwent intra-chain transfer rather than inter-chain transfer because the reaction with glycogen synthetase is
Fig. 2. Action of branching enzyme.

Inter-chain transfer

The sequential action of glycogen synthetase and branching enzyme on a multiply-branched polysaccharide (with equal numbers of A and B chains) is being represented diagrammatically as Fig. 2. However, as glycogen synthesis proceeds, the average chain length decreases progressively (102). To explain this, Smith (102) suggested that as glycogen synthesis proceeded, the branching enzyme was presented with progressively shorter chains. In Bruchovsky, these were acted on by the shorter chains, despite their lower affinity for the enzyme, the net result would be a transfer of shorter chain segments, with a consequent decrease in the average chain length if glycogen synthesis occurred.

Intra-chain transfer

Brown et al. (101) observed that the transfer of glucose from UDPG to glycogen took place entirely on the B-chains and indicated that the active action of the branching enzyme was confined to the outer part of the B-chain, whereas the transfer was more effective in the A-chain. When the branching enzyme was added to the system, it was not immediately transferred to glycogen synthetase.

α-1,4-linkages
↓
α-1,6-linkages
favoured with an outer chain of about 8 glucose units rather than with the shorter chains which would be produced by inter-chain transfer.

The sequential action of glycogen synthetase and branching enzyme, in the formation of a multiply-branched polysaccharide (with equal numbers of A-chains and B-chains) is being represented diagrammatically in Fig. 3. However, as glycogen synthesis proceeds, the average chain length decreases progressively (102). To explain this, Smith (102) suggested that as glycogen synthesis proceeded, the branching enzyme was presented with progressively shorter chains. If branching enzyme then acted on these latter chains, despite their lower affinity for the enzyme the net result would be a transfer of shorter chain segments, with a consequent decrease in the average chain length as glycogen synthesis occurred.

Brown et al (101) observed that the transfer of glucose from UDPG to glycogen took place almost entirely on the B-chains and indicated that the subsequent action of branching enzyme was confined to the outer part of the B-chains. Branching involved the transfer of a maltoheptaose unit to form an A-chain which was not immediately lengthened by glycogen synthetase.
Fig. 3. Sequential action of glycogen synthetase and branching enzyme.

A. \( \alpha-1,4 \)-linkages

B. \( \alpha-1,6 \)-linkages

(see D.J. Manners (97))
B. Glycogen Degradation

Glycogen phosphorylase (α-1,4-Glucoanorthophosphate glucoyltransferase; EC 2.4.1.1): The key step in the mobilization of glycogen is almost certainly the phosphorolysis of the polysaccharide to produce G-1-P. This is catalysed by the enzyme phosphorylase and the general reaction is as follows (75):

\[
\text{Glycogen (n glucose units)} + \text{PO}_4^{3-} \xrightarrow{\text{Phosphorylase}} \text{Glycogen (n-1 glucose units)} + \text{G-1-P}
\]

Rabbit muscle phosphorylase was isolated and crystallised by Green and Cori (103) and was observed to exist in "active" (a) and "inactive" (b) forms, which were interconvertible, and possessed molecular weights of 370 x 10^3 and 185 x 10^3, respectively (104).

Pyridoxal-5-phosphate has been identified as a prosthetic group of phosphorylase. Four moles are bound to form a and two to form b (105). The pyridoxal phosphate can be removed from the enzyme by acid hydrolysis; the process is reversible (106). Rats maintained on a diet deficient in pyridoxal exhibit a lowering of the total phosphorylase activity (107).

Phosphorylase b is activated by AMP; phosphorylase a, when freshly isolated from muscle, is independent of AMP for activity. The conversion of b to a occurs irreversibly in the presence of ATP, Mg_2^+ ions and the enzyme phosphorylase b kinase, (ATP: phosphorylase phosphotransferase; EC 2.7.1.38), according to the following scheme (105):

\[
2 \text{Phosphorylase b} + 4\text{ATP} \xrightarrow{\text{Phosphorylase b kinase}} \text{Phosphorylase a} + 4\text{ADP}
\]

Phosphorylase b kinase acts by phosphorylating two serine residues in phosphorylase b (a dimer) then dimerises to form a single tetrameric molecule of phosphorylase a (108).
Phosphorylase a can be converted to phosphorylase b in muscle by a phosphorylase a phosphatase (phosphorylase phosphohydrolase; EC 3.1.3.17), molecular weight 40,000 (109), which removes four phosphate groups from the tetramer (110).

\[
\text{Phosphorylase a} + 4\text{H}_2\text{O} \xrightarrow{\text{muscle phosphorylase \_a phosphatase}} 2 \text{phosphorylase b} + 4\text{P}_i
\]

Fischer et al (109) postulated that the a to b conversion did not proceed in an 'all-or-none' fashion but in a step-wise manner through partially phosphorylated intermediates. Sedimentation equilibrium studies confirmed this idea. Phosphorylase a phosphatase from bovine adrenal cortex was found to be inactivated by ATP; it could then be reactivated by adding ATP and Mg$^{2+}$ (111).

An additional complicating factor is that an inactive form of phosphorylase b kinase has been isolated from resting skeletal muscle, which can be activated by incubation with Mg$^{2+}$ ions and ATP (112). This process is also accompanied by phosphorylation of serine residues in the inactive kinase (112). The enzyme responsible for activation of the latter kinase is phosphorylase b kinase kinase (ATP: protein phosphotransferase; EC 2.7.1.37), and this has been isolated from rabbit skeletal muscle (113) and liver (114); it is cyclic -3',5'- AMP-dependent (113). The 'kinase kinase' links hormonal stimulation of adenyl cyclase and phosphorylase b kinase activation.

Inactive phosphorylase b kinase was also found to be activated by Ca$^{2+}$, in the presence of a kinase activating factor (115). The affinity of phosphorylase b kinase for both Ca$^{2+}$ and phosphorylase b, in muscle glycogen particles is modulated by other components of the protein-glycogen complex, in such a way that, in intact cells, the same concentration of free Ca$^{2+}$ ions released from the sarcoplasmic reticulum (2 x 10$^{-6}$ M.) stimulates both muscle contraction and glycogen breakdown (116). In the muscle protein-glycogen complex, phosphorylase
a phosphatase undergoes a reversible inhibition, requiring free Ca$^{2+}$, when phosphorylase is activated (117), that is, the same conditions triggering phosphorylase activation, which is observed only in intact complexes, occurs at the same concentration of Ca$^{2+}$ that triggers muscle contraction. The physiological significance in relation to muscle contraction is obvious. Conversion of the active phosphorylase b kinase to the inactive form is controlled by phosphorylase b kinase phosphatase in animal tissues (118). This catalyses the dephosphorylation of the b kinase. The various factors influencing the phosphorelease of glycogen can be summarised in diagrammatic form:

![Diagram of glycogen metabolism](image)

The inactive liver phosphorylase was found to differ from the skeletal muscle and heart enzymes in showing little or no activation by AMP, whereas the active enzyme was stimulated (119).
Stalmans et al (120) have recently established a close relationship between glycogen synthetase and phosphorylase (Fig.4). The action of synthetase phosphatase was preceded by a latency or lag period and it was postulated that the lag period was required for the action of an enzyme that allowed synthetase phosphatase to become active. However, they found that phosphorylase a inhibited liver synthetase phosphatase and that the lag period was the time required for the conversion of phosphorylase a into b. Phosphorylase b had a slight inhibitory action. Muscle synthetase phosphatase does not appear to be inhibited by phosphorylase (121).

**Glucose-6-phosphatase** (D-Glucose-6-phosphate phosphohydrolase; EC.3.1.3.9):G-1-P, a product of phosphorylase action on glycogen, is converted to G-6-P by phosphoglucomutase (see page 20). Glucose, for transport to the tissues, results in part from the hydrolysis of this phosphate by glucose-6-phosphatase.

Glucose-6-phosphatase is found in liver, kidney and small intestine (122) and is associated with the cell microsomal fraction (123). Swanson (124) first isolated the enzyme from liver but this preparation was relatively impure owing to associated lipids. No entirely satisfactory purification procedures are available at present. Duttera et al (125) found that phospholipid is required for maximal activity of glucose-6-phosphatase; treatment of the microsomes with phospholipase C (phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3) destroys 80-90% of the activity under conditions in which 70% of the phospholipid is hydrolysed.

Pyrophosphate, a by-product of UDPG synthesis, can also be used as a substrate by glucose-6-phosphatase (126,127) and in vivo the enzyme may have a dual role, i.e., as a phosphohydrolase and as a phosphotransferase.
Fig. 4. Inter-relationship of glycogen phosphorylase and glycogen synthetase.
Glucose + PP\textsubscript{i} $\xrightarrow{\text{Phosphotransferase activity}}$ G-6-P + P\textsubscript{i}

The latter property allows for an alternative route not involving kinases for the synthesis of G-6-P. Nordlie (128) has investigated both of these activities of glucose-6-phosphatase in microsomal preparations from liver, kidney and intestine of the rat, rabbit, guinea pig and man. In addition to PP\textsubscript{i}, a variety of other compounds such as CTP, ATP, ADP, GTP can serve as phosphate donor substrates (127).

The phosphohydrolase activity of the enzyme with G-6-P as substrate is competitively inhibited by PP\textsubscript{i} and various nucleoside di- and tri- phosphates and non-competitively inhibited by glucose. The glucose-6-phosphatase catalysed interconversions of glucose and G-6-P may be subject to these controlling factors in tissues (128).

Oligo-1,4→1,4-glucantransferase (α-1,4-glucan:α-1,4-glucan 4-oligo-glucantransferase; EC 2.4.1.24): Phosphorylase limit dextrins, as well as G-1-P, are obtained by the action of phosphorylase on glycogen. Oligo-1,4→1,4-glucantransferase transfers chains of glucose units from the outer branches of phosphorylase limit dextrin or glycogen to other parts of the molecule so as to expose single α-1,6-linked glucose residues at the branch points (129,130).

Whether phosphorylase itself, as originally postulated by Cori and Larner (131), can reduce an A-chain to a single glucose unit is still a matter of conjecture (132,133). Oligo-1,4→1,4-glucantransferase was found to preferentially transfer maltotriosyl,
and to a lesser extent, maltosyl, units from a donor to an acceptor substrate (130). In the process leading to the exposure of a glucose stub the donor will be an A-chain, and the receptor will be any terminal non-reducing glucose unit (75). In relation to the specificity of this transfer reaction, it should be noted that maltotriosyl units normally 'cover' the 1,6-linked glucose units in phosphorylase limit dextrin (132).

The reversibility of the reaction was confirmed by incubating glycogen, possessing $^{14}$C-labelled 1,6-linked glucose stubs, with glucantransferase. As a result, the stubs became 'covered' by unlabelled 1,4-linked glucose residues derived from other parts of the glycogen molecule (132).

Pure preparations of oligo-1,4\(\rightarrow\)1,4-glucantransferase have not, as yet, been made. Brown and Brown (133) were unable to separate rabbit muscle glucantransferase from amylo-1,6-glucosidase.

Amylo-1,6-glucosidase (Dextrin 6-glucanohydrolase; EC 3.2.1.33): Further degradation of glycogen after reaction with phosphorylase and oligo-1,4\(\rightarrow\)1,4-glucantransferase, can be effected with amylo-1,6-glucosidase and all three enzymes acting in concert can bring about the total degradation of glycogen (133) (see Fig.5). Amylo-1,6-glucosidase hydrolysates the single α-1,6-linked glucose units which remain at the branch points (134).

Purification of amylo-1,6-glucosidase has proved very difficult owing to glucantransferase contamination and it was originally suggested that glucantransferase activity was a function of amylo-1,6-glucosidase (135). However, evidence obtained by studies on a form of glycogen storage disease Type III showed that this was not correct. In this disease amylo-1,6-glucosidase is present in liver and muscle, but glucantransferase is absent (132). Taylor and Whelan (134) postulated that the two enzymes were normally bound as a multi-enzyme complex.
Purified rabbit muscle amylo-1,6-glucosidase/oligo-1,4→1,4-transferase complex can synthesise oligosaccharides, possessing α-1,6-linkages, using glucose, maltotetraose or α-Schardinger dextrin as substrates (136,137). The formation of isomaltose from glucose, in the absence of a polysaccharide and retention of the anomeric configuration, indicates that the synthetic mechanism goes via a glucosyl-enzyme intermediate rather than involving a polysaccharide-enzyme intermediate (136).

The various enzymic debranching reactions involving glycogen are shown in Fig.5 (134). The linear product is susceptible to complete phosphorolysis by phosphorylase. α-amylase (α-1,4-Glucan 4-glucanohydrolase; EC 3.2.1.1): Purely hydrolytic pathways may also result in glycogen degradation. α-Amylase, which is widely distributed in Nature (138), may well be involved in such processes. This enzyme requires bound calcium for activity and to prevent destruction by proteolytic enzymes (139) and chloride ions are also required (140).

Rat liver cellular distribution studies have shown α-amylase to be located in the microsomal fraction (141).

The action of α-amylase on starch-type polysaccharides is characteristically random and results in the hydrolysis of non-terminal α-1,4 glucosidic linkages in both the exterior and interior regions of the molecules (142). At low enzyme concentrations glycogen is hydrolysed to maltose, maltotriose and α-limit dextrins (containing α-1,6-branch linkages) (22). However, at higher concentrations of α-amylase, the maltotriose is slowly hydrolysed to maltose and glucose. The nature of the α-limit dextrins varies with the enzyme source. Each enzyme tends to produce different proportions of products. Olavarria and Torres (143) found that liver α-amylase acts on glycogen in two stages. Firstly, linear maltosyl oligosaccharides and maltose are produced from the outer branches of the
Fig. 5. Enzymic debranching of glycogen

Two distinct forms of α-amylose were observed to occur in rabbit muscle (124) and ox liver, in the liver (143, 146). The importance of α-amylose in the glycogen degradation in vivo remains obscure but it has been suggested that α-limit dextrin and α-limit dextrin are likely to be the main products of the action and not glucose (173).

Recent work with crystalline α-amylases from six different sources has shown that the glucose in the formation of α-limit and maltooligosaccharides is involved in a maltosyl fluoride intermediate (147). In addition, the amylase can transfer α-1,6-amylosyl linkages from maltosyl fluoride to C-4 carbonyl sites.

α-Glucosidase is glycohydrolase degrading oligosaccharides following α-amylolysis, further degradation of glycogen, and action of α-glucosidases.

Neutral α-glucosidase removes glucose units from the non-reducing ends of α-glucans, forming from disaccharides to polysaccharides. α-Glucosidases have been found in liver (148), kidney (149), spleen (150), skeletal muscle (145), small intestine (151), skin (152), livers (153), serum (154) and urine (155).

The livers of rat, guinea-pig, pig, ox and macaque rhesus monkey also contain several molecular forms of α-glucosidase. Here (156) has located a neutral form in the microsomal and soluble fractions and α-glucosidase in the lysosomal fraction ("neutral" and "acid" refer to the form of the enzyme). It is not known whether the neutral enzyme in the microsomal and soluble fractions is one enzyme or two distinct enzymes (156). The cellular localizations of α-glucosidases have been substantiated by other workers using rat kidney (157), rat liver (146, 150), and human liver (159). Contrary to the earlier findings of Here (156, 150), human liver was found to...
polysaccharides, and secondly, the central core of the molecule gives rise to branched products and maltose.

Two distinct forms of α-amylase were observed to occur in rabbit muscle (144) and one form in the liver (145,146). The importance of α-amylase in glycogen degradation in vivo remains obscure but it has been suggested that maltose and α-limit dextrins are likely to be the main products of the reaction and not glucose (75).

Recent work with crystalline α-amylases from six different sources has shown that the enzyme is able to catalyse the formation of maltose and malto- and maltosaccharides from α-D-glucopyranosyl fluoride; this involves a maltosyl fluoride intermediate (147). In addition, the enzyme can transfer α-D-glucosyl units from maltosyl fluoride to C-4 carbinol sites.

α-Glucosidases (α-D-Glucoside glucohydrolase; EC 3.2.1.20): Following α-amylolysis, further degradation of glycogen can occur by the action of α-glucosidases.

Mammalian α-glucosidases remove glucose units from the non-reducing ends of α-glucans, ranging from disaccharides to polysaccharides. α-Glucosidases have been found in liver (148), kidney (149), spleen (150), skeletal muscle (145), small intestine (151), skin (152), leucocytes (153), serum (154) and urine (155).

The livers of rat, guinea-pig, pig, ox and macacus rhesus monkey all contain several molecular forms of α-glucosidase. Hers (156) has located a neutral form in the microsomal and soluble fractions and an acid form in the lysosomal fraction ("neutral" and "acid" refer to the pH optima of the enzymes). It is not known whether the neutral activity in the microsomal and soluble fractions is one enzyme or two distinct enzymes (156). The cellular localisations of α-glucosidases have been substantiated by other workers using rat kidney (157), rat liver (148,158), and human liver (159). Contrary to the earlier findings of Hers (156,160), human liver was found to
contain a neutral α-glucosidase (150) but this enzyme was unable to hydrolyse glycogen. No neutral α-glucosidase could be detected in the livers of the gorilla (156) or rabbit (161).

The pH optima of the two forms of α-glucosidase depend on the source of the enzyme. Neutral α-glucosidases normally have pH optima which lie within the range 6.0 - 7.0, whereas the optima of the acid α-glucosidases are between 3.8 and 5.0 (75).

Purification of α-glucosidases has been effected by making use of the relative labilities of the neutral and acid forms to heat and to storage at neutral pH. The acid enzymes but not the neutral forms, survive treatment at pH 4.8 to 5.0 for 30 min. at 55°C. In addition, the neutral enzymes survive prolonged storage at 0°C, pH 7.0 whereas the acid enzymes do not (162-164). Abdullah et al (144) found three α-glucosidases in rabbit skeletal muscle (as well as amylo-1,6-glucosidase) and separated them, by salt fractionation, into a neutral enzyme and a mixture of acid and neutral enzymes. The latter two were then resolved by heating and neutral storage, respectively. Sephadex column chromatography has been used to purify acid and neutral α-glucosidases from rabbit muscle (165) and the livers of ox (166), dog (158) and rat (167,168). Fujimori et al (150) purified and crystallized acid α-glucosidase from bovine spleen.

Molecular weights of acid α-glucosidases differ according to the source of the enzyme. Calculations from sedimentation coefficients obtained by sucrose gradient studies give molecular weights, for acid enzymes, of 76,000-83,000 (169) and 109,000-114,000 (168) from rat liver, 97,000 from human kidney (169) and 107,000 from ox liver (166). Bruni et al (166) showed that ox liver acid α-glucosidase contained four -SH groups per molecule. Thiol group reagents did not affect the enzyme activity. The groups may be involved in stabilizing the protein molecule.

Acid α-glucosidase, in isolated rat liver cells, was observed to hydrolyse α-1,4-glucosidic bonds and to liberate glucose from glycogen.
as well as from malto-oligosaccharides (170). This enzyme is inhibited non-competitively by turanose; the neutral enzyme is not affected by this disaccharide (148). It has been postulated that acid $\alpha$-glucosidase and $\alpha$-amylase may act together to liberate glucose from glycogen thus (170):

\[
\text{Glycogen} \xrightarrow{\text{$\alpha$-amylase}} \text{oligosaccharides} \quad \text{acid $\alpha$-} \quad \text{Oligosaccharides} \xrightarrow{\text{glucosidase}} \text{glucose}
\]

However, acid $\alpha$-glucosidase can degrade glycogen directly and release glucose from the outer chains of the polysaccharide (146). The neutral $\alpha$-glucosidase appears to have a similar substrate linkage specificity to the acid form (75). However, human liver neutral $\alpha$-glucosidase has no action on glycogen although it rapidly hydrolyses malto-oligosaccharides to glucose (159).

Lukomskaya (163) showed that rabbit muscle acid $\alpha$-glucosidase was capable of synthesising oligosaccharides with $\alpha$-1,4-linkages from maltose. The neutral enzyme also synthesised oligosaccharides with $\alpha$-1,3-, $\alpha$-1,4- and $\alpha$-1,6- linkages from maltose, as well as from glucose. There have been several other studies showing that both the acid and neutral enzymes can catalyse glucosyltransfer reactions (152,158,159,168). Both $\alpha$-glucosidases were shown to have glucosyltransferase activities in the liver, but the neutral forms seem to be more effective in catalysing glucosyltransferase reactions than the acid enzymes (158). Human-tissue homogenates catalysed glucosyl transfer using maltose or maltotriose as donor to produce maltotriose and maltotetraose, respectively, at pH 7.1, but not at pH 4.1 (159). The transfer reaction was of minor significance compared with the prominent hydrolytic reactions (159).

One observation of possible physiological significance is that rat liver lysosomal acid $\alpha$-glucosidase can transfer labelled glucose from $^{14}$C-maltose to glycogen at pH 4.0; maltotriose, maltotetraose
and a branched trisaccharide of unknown structure are also formed (168). The importance of this and other transglucosylation reactions in vivo is not clear.

Purified acid α-glucosidase from rat liver lysosomes appears to have α-1,6-glucosidase as well as α-1,4-glucosidase activity (163) and both of these activities are lost in patients with Type II glycogen storage disease (171). Under certain conditions in vitro, purified lysosomal acid α-glucosidase can bring about total hydrolysis of glycogen to glucose (172) but the debranching of the polysaccharide by hydrolytic cleavage of its α-1,6-glycosidically linked branch point units is the rate-limiting step in the overall degradation of the polysaccharide.

Recent studies on purified rat liver lysosomal α-glucosidase, by Jeffrey et al (172), support the hypothesis that the enzyme has two catalytically active binding sites; one binding maltose and isomaltose and the other binding glycogen. Mutual inhibition studies using maltose and glycogen have indicated that each substrate appears to be a competitive inhibitor of the other and that the initial velocities of hydrolysis of these two substrates are influenced differently by cation concentration and by pH changes (168). Turanose was found to give mixed inhibition of the hydrolysis of the substrates and Jeffrey et al (172) postulated the existence of one separate inhibitory site in the enzyme molecule.

In view of the lysosomal location of acid α-glucosidase, it is unlikely to participate in the phosphorolytic pathway of glycogen degradation (160). It has been postulated that the main role of the enzyme in vivo is probably to degrade any glycogen which enters the organelle.
Hormonal Control of Glycogen Metabolism

Most animal hormones exert their effects by changing enzyme levels and consequently regulating the flow of metabolic pathways and the concentrations of certain metabolites in the cell. There are three general kinds of mechanisms by which hormones act, and these are:

(a) the stimulation of particular kinds of RNA synthesis,
(b) the stimulation of adenyl cyclase, resulting in an increased concentration of cyclic $3',5'$-AMP, and
(c) the alteration of rates of transport through cell membranes.

Hormones commonly influence the biochemistry of a specific tissue and this is related to the occurrence of special hormone receptors in these tissues. The receptors, in some cases, may be proteinaceous in nature.

Carbohydrate metabolism in tissues is regulated by the endogenous secretion of hormones, particularly at the enzyme level, which maintains a fine control over the blood glucose and tissue glycogen levels.

(i) Adrenaline and Glucagon

Adrenaline is synthesised and stored in chromaffin cells, from which it is released into the blood upon stimulation by the sympathetic nervous system. The chromaffin cells are found in the adrenal medulla and also along the aorta and in other locations. Adrenaline is a catechol-$\beta$-ethanolamine and only the natural L-isomer is fully active. In carbohydrate metabolism, the main effect of adrenaline secretion is an elevation of the blood glucose level.

Adrenaline stimulates the formation of cyclic $3',5'$-AMP from ATP by activating adenyl cyclase in rat skeletal muscle, resulting in an elevated phosphorylase activity and a decreased glycogen synthetase activity (see Fig.6) (173,174).
Fig. 6. Regulation of glycogen metabolism in liver tissues

Adrenaline
Glucagon

Glycogen synthetase D

ATP

Glycogen synthetase phosphatase
Glucagon is a small peptide (29 amino acid residues in the case of the hog hormone) secreted by the α-cells of the pancreatic islets, and, like adrenaline, its secretion results in an elevation of the blood glucose level; glucagon also stimulates gluconeogenesis from lactate or amino acids.

Glucagon is much more potent than adrenaline in stimulating hepatic adenyl cyclase (175). Bishop and Lamer (176) found that glucagon cancelled the activation of glycogen synthetase that had been induced in dog liver by injection of glucose and insulin, presumably by the stimulation of synthetase I kinase activity by increasing the levels of cyclic-AMP. Similar effects were observed with the livers of normal mice (177). Evidence has been presented for independent adenyl cyclase systems responsive to glucagon and adrenaline in the rat liver (178,179). Glucagon and adrenaline have also been shown to increase the intracellular content of cyclic-3',5'-AMP, via adenyl cyclase stimulation, in fat cells (180). Rail and Sutherland (181) noted that glucagon did not promote glycogenolysis in skeletal muscle, but did in liver, perhaps due to muscle cyclic 3',5'-AMP not responding to glucagon or due to glucagon not penetrating the muscle cells.

Free fatty acids inhibit a number of enzymes involved in glucose metabolism and hence it should be noted that both glucagon and adrenaline produce an increase in triglyceride lipase activity in the rat (182) and this is accompanied by an increase in free fatty acid and glycerol levels in the plasma. Rizak (183) stated that the rise in activity of triglyceride lipase was due to an increased production of cyclic-3',5'-AMP. Similar conclusions were reached from the studies of adrenaline action on adipose tissue by Butcher et al (184).

The main effects of free fatty acids on carbohydrate metabolism, i.e., inhibition of phosphorylation of glucose and fructose and...
inhibition of pyruvate kinase, are shown in Fig. 7 (185). The net
effect of glucagon and adrenaline on carbohydrate metabolism is
an elevation of the blood sugar level as the hormones inhibit the
glycolysis sequence and promote G-6-P formation, which is dephos-
phorylated by glucose-6-phosphatase.

(ii) Adrenocorticotropic hormone (ACTH).

ACTH is believed to be produced and stored in the basophilic
cells of the anterior pituitary, and is a straight-chain polypeptide
containing 39 amino acid residues with a molecular weight of 4,500.
The direct effect of ACTH on the adrenal gland is to promote the
production of corticosteroids.

Adenyl cyclase in isolated fat cells is stimulated by ACTH,
and this results in an increase in the intracellular content of
cyclic-3',5'-AMP (180). A divalent ion, such as Mg$^{2+}$, is necessary
for adenyl cyclase activity in fat cells (186) and the effect of ACTH
is to increase the affinity of the enzyme for Mg$^{2+}$.

In adrenal glands, ACTH also produces an increase in the level
of cyclic -3',5'-AMP, which, in this tissue, inactivates phosphorylase
a phosphatase (111). A similar reaction occurs in pigeon breast
muscle extracts (187). Hence, ACTH indirectly activates phosphorylase
and favours glycogen degradation in these particular tissues. This
reaction has not been observed in the liver.

(iii) Thyroid hormones

The thyroid gland elaborates iodinated derivatives of tyrosine,
mainly thyroxine and also triiodothyronine. These hormones have two
important effects. One is to increase the metabolic rate and the
other, to promote normal development of the growing organism.

Injection of noradrenaline into rats, like adrenaline, activates
phosphorylase and in the former case the activation is enhanced by
pretreatment of the rats with triiodothyronine. It has been suggested
that this is due to an increase in adenyl cyclase activity (188) or to
inhibition of phosphodiesterase activity (orthophosphoric diester
Fig. 7. The hormonal control of key gluconeogenic and key glycolytic enzymes.
phosphohydrolase; EC 3.1.4.1) (169), both of which would favour higher concentrations of cyclic-3',5'-AMP.

In triiodothyronine-treated rats there is a reduced capacity for glycogen deposition in the liver which may be caused by induction of glucose-6-phosphatase activity, and hence a lowering of the G-6-P concentration and a decrease in glucose utilization via the glycolytic pathway (190).

Thyroidectomy in rats was found to diminish glucose synthesis from lactate in the liver, but this was restored to normal after treatment with triiodothyronine (191). The reduced rate of glucoseogenesis in hypothyroid rats was suggested to result from the reduced activity of pyruvate carboxylase and of phosphoenolpyruvate carboxykinase.

(iv) Thyroid stimulating hormone (thyrotrophin; TSH).

TSH is a linear polypeptide containing a carbohydrate moiety. The carbohydrate residues are in a single oligosaccharide unit which is linked to the polypeptide by a covalent bond. TSH is produced in the anterior pituitary basophil cells and its function is to control the activity of the thyroid gland.

TSH was found to produce a large and longlasting stimulation of adenyl cyclase activity in bovine thyroid slices (192) and in isolated fat cells (180) but there is some doubt regarding the effect of the increase in cyclic-AMP on phosphorylase activation in these particular tissues. Gilman and Rall (193) found no significant changes in phosphorylase a levels in bovine thyroid slices incubated with TSH, whereas Butcher and Serif (195) observed a three-to five-fold increase in phosphorylase a in dog thyroid slices that had been incubated with the hormone. The pattern of thyroid response to TSH treatment may vary from one species to another or the hormonal effects may result from different mechanisms in different species. (195). This topic has been reviewed by Schell-Frederick and Dumont (195).
TSH stimulates lipolysis in rat adipose tissue and it appears to be mediated by cyclic-3',5'-AMP (196).

\( \text{Insulin} \)

Insulin, a large peptide, is formed in the \( \beta \)-cells of the pancreatic islets and consists of an acidic (A) and a basic (B) chain usually containing 21 and 30 amino acids, respectively. The two chains are interconnected by two disulphide bridges. The general effects of insulin secretion consists of a lowering of blood glucose levels, an increase in the rate of glucose transport into skeletal muscle and adipose tissue, an increase in glycogen synthesis and a lowering of the concentration of cyclic-3',5'-AMP.

Butcher et al (197) showed that insulin lowered the intracellular cyclic-3',5'-AMP level in liver and in epididymal fat pads of rats. Bishop and Larner (176) observed that when insulin was administered to dogs, liver glycogen synthetase was activated, whereas phosphorylase was inactivated. They attributed both enzyme changes to a fall in cyclic-3',5'-AMP levels.

The mechanism by which insulin lowers cyclic-3',5'-AMP levels is, as yet, not clearly understood. Insulin does not affect isolated adenyl cyclase from fat cells (198,199). Senft et al (200) observed an increase in phosphodiesterase activity in the adipose tissue and liver of fasted rats which had been injected with insulin. They postulated that the rise in phosphodiesterase activity would lower the levels of cyclic-3',5'-AMP.

In rabbit skeletal muscle, it appears that the insulin effects on glycogen synthetase activity is not mediated by changes in cyclic-3',5'-AMP levels (201,202). Insulin appears to control synthetase I kinase activity under conditions where the tissue levels of cyclic-3',5'-AMP do not decrease (202). It was postulated that insulin and adrenaline acted on the synthetase I kinase site in different ways. Insulin is thought to inactivate the kinase and thus increase the
proportion of synthetase I. Adrenaline, on the other hand, increases cyclic-3',5'-AMP levels which stimulates the kinase and in turn promotes the I- to D conversion. It was assumed that the proportion of synthetase I that is observed at any one time represents a balance between the activity of the phosphatase catalysing the D- to I conversion and the kinase catalysing the I- to D conversion. Such a balance could be tilted by insulin on adrenaline.

Synthetase I kinase measured without added cyclic-3',5'-AMP was decreased in muscle extracts of insulin-injected rats, whereas in the presence of excess cyclic-3',5'-AMP, the level remained unchanged (203). If the physiologically active form of synthetase I kinase is the cyclic-3',5'-AMP-independent form, the effect of insulin on glycogen synthesis could be explained by the conversion of an active to an inactive form of synthetase I kinase in muscle, that is, to a form more dependent on cyclic-3',5'-AMP.

A tentative suggestion has been made that there is a positive relationship between the activity of branching enzyme and insulin (97). The evidence is as follows: humans having "brittle" diabetes when treated with insulin show hepatomegaly with an increased glycogen content (8-9%), and the glycogen has a normal degree of branching (204). Hence it is likely that the activity of the branching enzyme increases proportionately to the activity of glycogen synthetase (97).

The properties of hexokinase II, from ascites tumour cells, differ depending upon whether the enzyme is free or bound to mitochondrial membranes (63). For example, the specific activity of the latter form is greater and the free enzyme is more sensitive than the bound enzyme to product inhibition by G-6-P. The effect of insulin on blood sugar levels could therefore be explained if the hormone was responsible for the binding of soluble hexokinase to mitochondria (205).
In support of this theory, alloxan diabetic rats show a decrease, from 46% to 11%, in the proportion of mitochondrial-bound hexokinase in lactating mammary gland and total hexokinase activity decreases (206). In addition, incubation of rat epididymal fat pad with insulin results in an increase in the percentage of mitochondrial-bound hexokinase (207).

Acute insulin deprivation by anti-insulin serum injection produces a 30% decrease in rat liver glucokinase activity (208). An accelerated rate of enzyme inactivation rather than an inhibition of enzyme synthesis appears to be responsible for this effect (see Fig. 8) (209).

Morgan et al (210) observed that insulin increased the rate of transport of D-glucose across the cell membrane in heart muscle probably by making a greater number of glucose transport sites available on the membrane by altering its structure (211). Phospholipase C had a similar effect.

Insulin also acts as an inducer of important glycolytic enzymes and a suppressor of key gluconeogenic enzymes; this has been reviewed by Weber (212) and the effects are summarised in Fig. 9.

(vi) Glucocorticosteroids (glucocorticoids)

Glucocorticoids are produced by the adrenal cortex and promote the deposition of glycogen in the liver, produce glucose from amino acids and retard the oxidation of glucose; they are thus diabetogenic and act as insulin antagonists. The structure of glucocorticoids is based on a pregnene ring structure:

![Chemical structure of glucocorticoid](image)

Cholesterol is the precursor of the glucocorticoids.
Fig. 3. Regulation of the glucose to G-6-P pathway in insulin-sensitive tissues

Muscle and adipose tissue

Glucose $\rightarrow$ Glucose

Hexokinase

Insulin

Glycogen

G-6-P $\rightarrow$ ATP + Pyruvate

Liver tissue

Glucokinase

Glucose $\rightarrow$ Glucose

NADPH + Pentose-Phosphate

Outside cell membrane

Inside cell membrane

Inhibition

Activation

Induction

Insulinogenesis

Pentose acids
Fig. 9. Induction and suppression of liver enzymes by Insulin

Conversion of blood glucose to liver glycogen (216). This was also observed in diabetic animals (214) and was therefore said to be insulin-independent. However, later work (213) has shown that this process is insulin-dependent.

Glycogen, a synthetic glucocorticoid, when injected into the liver, acts as a phosphatase and stimulates the synthesis of the enzyme (216). These observations explain the increased activity of glycogen phosphorylase by injection of glycogen into the liver after glucocorticoid administration (217). These observations were given by the in vitro study of glycogen phosphorylase (218). Insulin, which in this way can be activated by glucocorticoids, which favour conversion of phosphofructokinase to the non-inductive form, can increase glycogen synthesis and hence glycogen (216).

The time course of these metabolic changes in the liver and the conversion of glycogen, synthetase to the more active form and hence glycogen is shown in Fig. 7 (see Ashmore and Weber (185)).

† induction effects.
○ suppression effects.

(see Ashmore and Weber (185))
Glucocorticoids have been observed to increase the rate of conversion of blood glucose to liver glycogen (213). This was also observed in diabetic animals (214) and was therefore said to be insulin-independent. Bergamini et al (215) have claimed that this process is insulin-dependent however.

Prednisolone, a synthetic glucocorticoid, when injected into mice, produced an increase in liver phosphorylase $\alpha$-phosphatase activity probably by initiating a more rapid synthesis of the enzyme (216). These observations explain the lowered activity of phosphorylase, by conversion of $\alpha$ to $\beta$, in liver after glucocorticoid treatment of animals (177) (see Fig. 6).

Phosphorylase $\alpha$ is a strong inhibitor of liver glycogen synthetase D phosphatase, the reaction is reversed by AMP and Mg$^{2+}$ ions (120). Phosphatase, which is inhibited in this way, can be reactivated by glucocorticoids which favour conversion of phosphorylase $\alpha$ to the $\beta$ form, which is non-inhibitory. This in turn will favour the conversion of glycogen synthetase D to the more active I form and hence glycogen synthesis (120, 216).

Daw et al (217) observed a diurnal variation in the cardiac glycogen concentration which was highest in the morning and lowest in the evening. Secretion of adrenal steroids follows a similar pattern and it was suggested that one factor responsible for these diurnal variations might be the diurnal fluctuations in plasma corticosteroid concentration.

The effects of glucocorticoids on gluconeogenic enzymes are shown in Fig. 7. (For reviews see Weber (212) and Ashmore and Weber (183)).

Suzuki et al (236) showed that glucagon administration to rats and dogs resulted in liver glycogen breakdown and an associated elevation of blood lactate, but not blood glucose and they suggested that the response to endogenous secretion of glucagon would be similar.
Glycogen Storage Diseases

In the group of diseases associated with abnormalities in glycogen metabolism, the elucidation of specific enzymic defects has progressed to a stage where classification on an aetiological basis has become possible. Since van Creveld in 1928 (218) described the first disorder of this type, numerous other cases have been reported and each has a confirmed or probable enzyme deficiency resulting in the accumulation of glycogen. Fig.10 (219) shows the sites of enzyme lesions causing excessive glycogen deposition in the tissues. Glycogen storage diseases have been extensively reviewed in the literature (220-222).

Type I Disease

This is the most common form of glycogen storage disease and was named after von Gierke who described the first case (223). Cori and Cori (224) found the disease was due to deficiency of the microsomal enzyme glucose-6-phosphatase and this results in glycogen accumulation (up to 16%) particularly in the liver and kidneys. The accumulated glycogen is of normal structure, and has an average chain length of 14.5 glucose units (225). Byron (47) observed that the cytoplasmic granules of glycogen in the cells of liver and kidney were of normal size. A deficiency of glucose-6-phosphatase in the intestinal mucosa has also been reported to occur in Type I cases (226).

Other patients lacking glucose-6-phosphatase have been described (227), some showing a tendency to degrade glycogen to lactate instead of glucose (228), thereby by-passing the glucose-6-phosphatase step. Also, these patients cannot convert galactose and fructose into glucose, but they convert these sugars into lactate (229).

Sarcione et al (230) showed that glucagon administration to rats and dogs resulted in liver glycogen breakdown and an associated elevation of blood lactate, but not blood glucose and they suggested that the response to endogenous secretion of glucagon would be similar.
Fig. 10. **Glycogen Storage Diseases**

**Type IV Brancher deficiency amylopectinosis**

- Glycogen → Branched glycogen
- Oligosaccharides → Glucose
- G-1-P

**Type II Pompe's disease (α-glucosidase deficiency)**

- G-6-P / Glucose
- G-1-P

**Type III debrancher deficiency limit dextrinosis**

- G-6-P
- F-6-P → F-1,6-diP

**Type V & VI**

- Glycogen → Branched glycogen

**Type VII Glucose-6-phosphatase deficiency glycogenosis**

- X marks the site of enzyme lesions causing excessive glycogen deposition

(1) Hexokinase or glucokinase

(2) Phosphoglucomutase

(3) UDPG-glycogen-transglucosylase

(4) Amylo-1,4→1,6-transglucosidase

(5) Phosphorylase

(6) Amylo-1,6-glucosidase

(7) Glucose-6-phosphatase

(8) α-glucosidase

(9) α-amylase

(10) Phosphofructokinase
It was concluded (230) that glucagon, in Type I glycogen storage disease, was responsible for increased lactate formation and that the Type I glycogen storage disease was not a consequence of the glucose-6-phosphatase deficiency per se, but that increased glycogen synthesis must play a role in accounting for the excessive liver glycogen content. Occasional cases have been reported in which glucagon administration gave rise to a normal response, that is, an elevation of the blood glucose level (219). It was suggested that this was due to glucagon increasing the activity of amylo-1,6-glucosidase with the consequent liberation of glucose from phosphorylase limit dextrin stubs, thus by-passing the glucose-6-phosphatase step. Adrenaline has also been found to elevate blood lactate levels, but apparently it does not reduce the hypoglycaemic condition observed in Type I glycogen storage disease patients (231).

Some cases of Type I storage disease have been reported to have elevated blood uric acid levels (229) and this is thought to be due to an increased conversion of G-6-P to 5-phosphoribosylpyrophosphate and uric acid (232).

Death in infancy due to this disease may occur, but patients who survive beyond the fourth year of life tend to have milder clinical manifestations, even though the glycaemic response to glucagon and adrenaline and the enlarged liver remains (220). With age, the patients tend to become normoglycaemic (233) and it was suggested that this was caused by a decreased insulin output as these cases show a diminished insulin response to ingestion of glucose.

Cori and Cori (224) could not explain the absence of glucose-6-phosphatase activity in Type I disease by a cofactor deficiency or the presence of an inhibitor. As the enzyme defect appears concurrently in the liver and the kidney, it has been suggested that it is controlled by the same gene (234) and that the disease is inherited as an autosomal recessive gene trait (226).
Boley et al (235) recommend total portal venous diversion in early infancy in children with severe hypoglycaemia before permanent cerebral damage could occur. If this is carried out, clinical improvement is observed, although the liver glycogen level is not reduced.

**Type II Disease**

Type II glycogen storage disease is also known as generalized cardiomegalic glycogenosis or Pompe's disease (236). The main clinical symptomatology concerns the cardiac and skeletal muscles and liver (237,238,239). Survival beyond the first or second year of life is rare.

Hers (160,240) discovered that in this type of glycogenosis, there was an absence of acid α-1,4-glucosidase which was normally present in human liver, heart and muscle (160). Lejeune (148) showed that the acid α-1,4-glucosidase was localised in the lysosomes of rat liver. Since the initial discovery by Hers, acid α-glucosidase has been found to be deficient in many other tissues from Type II cases, namely: leucocytes (241,242), thyroid and adrenal tissue (243), fibroblast cell cultures (244,245), amniotic fluid, fluid cells and cultivated fluid cells (246,247).

The deposition of glycogen of normal structure in Type II glycogenosis reaches mean values of 11% for skeletal muscle, 6.5% for cardiac muscle and 9% for liver (248). However, Smith et al (249) reported isolating glycogen having abnormally short outer branches and suggested that this was due to an elevated level of neutral α-glucosidase. Baudhuin et al (50) observed a dual localisation of glycogen in the liver of a child with Type II glycogenosis. Part appeared in the form of rosettes freely dispersed in the cytoplasm, as in normal liver, but the large proportion was segregated in vacuoles surrounded by a single membrane; the lysosomes. These observations have been substantiated by other workers (49,221,248,250,251). The concept of an inborn lysosomal enzyme defect explains the intralysosomal
accumulation of glycogen in many tissues. Hug et al. (252), on the basis of interpretations of electron micrographs of liver tissue, observed two forms of cytoplasmic glycogen: a monogranular and a multigranular form. These workers suggest that lysosomes are required to ingest the monogranular form and that they must contain α-glucosidase to degrade it. The multigranular form is subject to degradation to glucose by the enzymes involved in glycogenolysis. This latter form also accounts for the lack of hypoglycaemia in Type II patients since it is mobilized during normal responses to glucagon (252) and adrenaline (251,253). To account for the accumulation of glycogen in two locations (lysosomal and cytoplasmic), it has been postulated that more than one enzyme is deficient in Type II glycogenosis (250,254).

In severe cases of the disease, glycogen is deposited in the nervous system (242,243,249,255) which helps explain the occurrence of mental retardation and neuromuscular conditions. Mild forms of the disease have been observed in adults (256) and in one case a lowered acid α-glucosidase activity was associated with hypothyroidism (257).

Hug and Schubert (250) reported that intravenous administration of a fungal enzyme mixture, containing α-1,4- and α-1,6-glucosidase activities, over a period of 18 days, to a patient having Type II glycogenosis results in a decrease in the liver glycogen content. However, an immune response to such an administration of fungal proteins limits the usefulness of this therapy (259). Intramuscular injection of a similar mixed enzyme preparation to a Type II case, over a 7 day period of time, did not decrease the liver and skeletal muscle glycogen levels (260).

The use of lipid spherules or liposomes containing fungal amylo-glucosidase activity have been used in an attempt to lower the
glycogen level in rat liver (261). The glycogen level did not fall even though there was an increased glucosidase activity associated with the mitochondrial-lysosomal fraction.

Vitamin A therapy has also proved unsuccessful in alleviating the glycogen storage (262).

Brown et al (171) have concluded that lysosomal α-glucosidase possesses α-1,6-glucosidase activity as well as α-1,4 since, both activities were missing in all the tissues with Type II glycogen storage disease that were examined.

A recent biochemical finding has been the possibility of achieving a strain of cats with suggestive Pompe's disease (263), which obviously would greatly facilitate further research.

**Type III Disease**

Type III glycogen storage disease was shown by Illingworth et al (264) to be due to a deficiency of amylo-1,6-glucosidase. Increased concentrations of glycogen with short outer chains are found in the liver, myocardium, skeletal muscle (265) and erythrocytes (229,266). In the skeletal muscle, glycogen accumulates beneath the sarcolemmal membrane, between myofibrils and between filaments (267). This results in disorganisation, degeneration and replacement of myofilaments in many fibres.

It is not uncommon to encounter cases where increased glycogen storage restricted to the liver and here Hers (263) has observed enzyme deficiency in the liver but not in the muscle. Illingworth and Brown (269) have made similar observations. Hence, Type III glycogen storage disease can be subdivided into (268):

- **IIIA** - enzyme defect in liver and muscle.
- **IIIB** - enzyme defect in liver only.

Analyses of the deposited polysaccharides indicated that there are two types. One with a Cori-Larner type structure with short chains of less than four glucose units (270) and the other having the Walker-Whelan structure with longer outer chains of 4-5 glucose units (271).
This suggests that two types of limit dextrinosis exist, one with amylo-1,6-glucosidase deficiency and the other due to a deficiency of oligo-1,4 → 1,4-glucantransferase and amylo-1,6-glucosidase (271).

The clinical manifestations of the disease include hepatomegaly and fasting hypoglycaemia, but these are less severe than in Type I storage disease. Glucagon or adrenaline produce a normal or near-normal hyperglycaemic response in these cases (272) and this may serve as a diagnostic aid in differentiating Type I from Type III glycogenosis.

Type I and Type III glycogen storage diseases may be associated in the same family (225,270); for example, a pair of siblings, one having diminished glucose-6-phosphatase and the other Type III glycogenosis, have been observed. Two siblings, one with Type I and the other with Type III, both with high erythrocyte glycogen levels have also been reported (273).

Waaler et al (274) obtained results that were compatible with the assumption that Type III glycogen storage disease is inherited by an autosomal recessive trait.

Type IV Disease

This disease was found to be due to the deficiency of the branching enzyme amylo-1,4 → 1,6-transglucosidase (275). Very few cases have been reported (222). The deposited glycogen exhibits an abnormal structure with very long outer chains (on average 14.7 glucose units) and relatively few branch points, i.e., it resembles amylopectin. Glycogen becomes deposited in the liver, myocardium, skeletal muscle, spleen, lymph nodes and erythrocytes (222).

The accumulation of a branched polysaccharide in the absence of a branching enzyme has not been satisfactorily explained. There are two possibilities (97):

(i) that two branching enzymes exist in the liver, only one of which is measured during analysis.

(ii) that the branching enzyme is present at the foetal stage and then decreases at birth.
Metabolism of the existing branched polysaccharide would then be restricted mainly to elongation by synthetase and degradation by phosphorylase. The total number of glycogen molecules remaining would be approximately constant.

Another explanation for the occurrence of branch points in the glycogen produced in this disease is that the polysaccharide may be synthesised via reversal of the debranching enzyme system, amylo-1,6-glucosidase/oligo-1,4→1,4-glucan transferase (276, 277). It has been shown, using a rabbit muscle enzyme system, that this reversal can occur (276).

Type V Disease

Type V glycogenosis is characterised by a deficiency of glycogen phosphorylase in skeletal muscle (278) although glycogen phosphorylase b kinase and glycogen phosphorylase phosphatase activities are normal (279). The deposited glycogen has a normal structure (222, 279) with respect to degree of branching and average outer chain lengths.

The clinical manifestations include the ease of fatigability, muscular cramping on exercise, general weakness, reduced muscular strength, and wasting of muscles after the age of 40 years (280).

Patients show a normal hyperglycaemic response to adrenaline and glucagon and this shows that phosphorylase deficiency does not extend to the liver (281).

Examination of one family has shown that this deficiency is genetically determined and is due to an autosomal recessive gene (280).

Type VI Disease

Patients suffering from Type VI glycogen storage disease have reduced glycogen phosphorylase activity in the liver and leucocytes (229, 240).

The glycogen is of normal structure and the patients suffer from hypoglycaemia and the hypoglycaemic response to glucagon and adrenaline, however, is subnormal (282). Hers (283) has concluded
that this type of glycogenosis is probably the most common and points out that the distinction between normal and low glycogen phosphorylase activities is not very clear cut but Type VI glycogen storage disease may be a consequence of some other more primary defect.

Huijing (284,285) subdivided Type VI glycogenoses into VIa and VIb. In Type VIa, there is a diminished glycogen phosphorylase b kinase activity in leucocytes and this is responsible for the low phosphorylase activity. This defect may be due to a defective kinase or an excessively rapid inactivation of the kinase (285). In Type VIb, there is a low glycogen phosphorylase activity in liver and in leucocytes but the phosphorylase b kinase activity is normal.

Phosphorylase b kinase deficiency has been shown to follow a dominant pattern of inheritance (284,286), while the form of the disease associated with glycogen phosphorylase deficiency follows an autosomal recessive pattern of inheritance (286).

Patients with Type VI glycogen storage disease show a subnormal hyperglycaemic response to glucagon or adrenaline administration (282). However, Hug et al (287) found a normal glucagon response when administered to Type VI patients having liver phosphorylase b kinase deficiency. The reason why such patients respond differently to glucagon is unknown.

**Type VII Disease**

The enzyme defect in this instance has been identified as an almost complete lack of muscle phosphofructokinase, which results in deposition of glycogen of normal structure (288) and accumulation of hexose monophosphates.

The clinical manifestations are ease of fatiguability, intolerance to exercise, marked weakness and stiffness in muscle groups which have been exercised.

It seems likely that the Type VII glycogen storage disease is due to a rare defect in an autosomal recessive gene since the disease
was reported in the offspring (siblings of both sexes) produced by a consanguinous mating (287).

Hug et al (252) reported that several Type VI patients responded differently to their reactivity towards glucagon and adrenaline and considered these cases to be new forms of glycogen storage disease which they termed Types VIII and IX. However, Hers and van Hoof (289) have doubted the existence of these two additional types. They stated that the unreactivity of cases of glycogenosis to adrenaline and glucagon were ill-understood and noted that exceptions to the rule had occasionally been observed in all types of the disease, particularly in Type VI.
**Lysosomes**

Lysosomes were first recognised as distinct subcellular particles by De Duve et al. in 1955 (290) and since this time their structure and function (formation, latency, stabilisation, labilisation, autophagy, effects of drugs and toxic reagents, etc.) have been extensively studied (291-295).

These organelles are found in almost all tissues and basically they consist of numerous hydrolytic enzymes which are surrounded by a single lipoprotein membrane. A more reliable morphological criterion obtained by electron microscopy, is that most lysosomes possess an electron-lucid rim or halo beneath their limiting membrane so giving a double membrane appearance. Often lysosomes are round or oval and are relatively dense and homogeneous. Sometimes they appear as multivesicular bodies. The mean diameter and density of these particles is 0.4μ and 1.19g cm⁻³, respectively, but these values depend on their developmental state. (see Fig.11) (293). Secondary lysosomes, and related autophagosomes and residual bodies (see Fig.11) have a varied structure, and often contain glycogen, concentric myelin figures, microbodies or cellular components.

The lysosomes contain numerous enzymes which function best in weakly acidic solutions (pH 3.0-6.5) and it has been suggested that the pH of secondary lysosomes, in which digestion occurs, is low and favours activity of the hydrolytic enzymes.

One of the functions of lysosomes is intracellular digestion (the stages of digestion are shown in Fig.11). Materials are ingested by a process of endocytosis, enclosed in phagosomes and then these fuse with primary lysosomes to form secondary lysosomes. Here, the hydrolytic enzymes degrade proteins, nucleic acids, carbohydrates and lipids, and any indigestible material remains in residual bodies. Other processes in which lysosomes are thought to be involved include autolysis (death and breakdown of tissues inside or outside
Fig. 11. Diagrammatic representation of the various components of the lysosomal system in the cell. Acid hydrolases are represented by crosses.

- Ph: Phagosome (Heterophagosome)
- Sl: Secondary lysosomes (Heterolysosome)
- Pl: Primary lysosome
- Av: Autophagic vacuole (Autophagosome)
- M: Mitochondrion
- Rer: Rough endoplasmic reticulum
- Rb: Residual body (Telolysosome)
- Pol: Post lysosome (no enzymes; accumulated undigested material)
- Ng: Nascent granule
- G: Golgi apparatus
- N: Nucleus
- R: Ribosomes

Endocytosis of particles

Exocytosis

Transport

Synthesis

Secretion of hydrolases
the body), uptake of drugs and toxic materials, autophagy (digestion of part of their own cytoplasm), carcinogenesis and mechanisms involving inflammation and immunology.

Lysosomes have been separated from cellular homogenates by:-
(1) differential centrifugation techniques (159, 296);
(2) sucrose-density gradient centrifugation of a light mitochondrial fraction (290) with or without prior administration of Triton WR1339 to animals (a non-ionic detergent) (296);
(3) zonal centrifugation using sucrose-density gradients (297, 298).

The rupture of lysosomes can be effected by many means. For example, by homogenisation, use of hypotonic media, freezing and thawing, sonication and use of lecithinase (phosphatide acyl-hydrolase; EC 3.1.1.4), proteases, detergents and carbon tetrachloride by incubation at pH 5.0, 37°C. (299).

Steroids have been shown to stabilise lysosomes in vitro and in vivo possibly by reaction with the surface lipid layers of the membranes (300). Weissmann and Dingle (301) proposed that corticosteroid stabilisation of lysosomes was the basis of the anti-inflammatory action of these compounds. The concentration of the steroid is a critical factor in steroid-lysosome interaction and most steroids stabilise lysosomes at pharmacological concentrations ($10^{-4}$ - $10^{-6}$M) but lyse them at higher concentrations ($10^{-2}$M) as shown by the release of lysosomal acid phosphatase (302).

Weissmann and Dingle (301) showed that vitamin A and cortisone had antagonistic effects on lysosomes in vitro and in vivo. Vitamin A caused the release of enzymes from lysosomes, but pretreatment of rats with cortisone retarded this release. Similar observations were noted using vitamin A and hydrocortisone hemisuccinate (303).

Considerable evidence (304) supports the membrane-action hypothesis of steroid-lysosome interaction proposed by Willmer (305)
despite the use of high concentrations of steroids. De Duve et al (299) suggested that steroids exerted their effects by replacing cholesterol molecules at certain sites in the lysosomal membranes and thus modified the structure and physical properties of the membrane. It has been found that steroids interact with artificial lipid spherules (liposomes) to produce changes in their permeability, (as shown by marker dyes) which resembled steroid-induced changes in natural membranes (306) (307).
Results and Discussion

The original purpose of this research on rat liver α-glucosidases was:

(i) to purify and study the properties of the enzymes; this work was curtailed, however, owing to the publication of a detailed study on acid α-glucosidase which appeared during the course of this present investigation (168, 172) and an early decision to concentrate on (ii) (below) in view of some very interesting initial observations that had been made.

(ii), to investigate the possible hormonal control of acid α-glucosidase activity in vitro and in vivo.

Purification and properties of rat liver α-glucosidases

Ammonium sulphate fractionation of centrifuged rat liver homogenates resulted in a 30-60% fraction in which both acid and neutral α-glucosidases were evident. In agreement with previous findings of Torres and Olavarría (158) (Fig. 12).

In an attempt to separate acid and neutral α-glucosidases, and in particular to purify the former, two purification steps were included prior to the gel filtration procedure. A 105,000 g supernatant solution from a rat liver homogenate was subjected to acid precipitation (pH 4.8) and then a 30-60% ammonium sulphate fraction of the resulting supernatant was taken. A sample of this ammonium sulphate fraction was reconstituted in buffer, dialysed and then applied to a Sephadex G-100 column which was eluted with 0.1M-sodium-acetate buffer at pH 4.8. Enzyme activity measurements were made at pH 3.4 (acid α-glucosidase) and at pH 7.3 (neutral α-glucosidase). The resulting elution profile is shown in Fig. 13. Two well separated peaks of activity were obtained; one corresponding to acid α-glucosidase (peak I) and the other (peak II) to neutral α-glucosidase.

The purification procedure for the acid α-glucosidase is summarised in Table I. Only a 4-fold purification resulted after gel filtration. No better purification resulted in nine independent experiments.

The pH optima of the activities in peaks I and II were checked and the results are shown in Fig. 14. Peak I had an optimum of
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(i) Purification and properties of rat liver α-glucosidases

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In an attempt to separate acid and neutral α-glucosidases, and in particular to purify the former, two purification steps were included prior to the gel filtration procedure. A 105,000 g supernatant solution from a rat liver homogenate was subjected to acid precipitation (pH 4.2) and then a 30-60% ammonium sulphate fraction of the resulting supernatant was taken. A sample of this ammonium sulphate fraction was resuspended in buffer, dialysed and then applied to a Sephadex G-100 column which was eluted with 0.1M-sodium acetate buffer at pH 4.8. Enzyme activity measurements were made at pH 3.6 (acid α-glucosidase) and at pH 7.5 (neutral α-glucosidase). The resulting elution profile is shown in Fig.13. Two well separated peaks of activity were obtained; one corresponding to acid α-glucosidase (peak I) and the other (peak II) to neutral α-glucosidase.

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Fig. 12. Ammonium sulphate fractionation of rat liver homogenates.

- - , acid α-glucosidase activity (pH 3.6)
- - , neutral α-glucosidase activity (pH 7.5)
Fig. 13. Sephadex G-100 chromatography of rat liver α-glucosidases.

-○-, protein; -x- and -Δ- are acid and neutral α-glucosidase activities, respectively; ---x--- and ---Δ--- are acid and neutral α-glucosidase specific activities, respectively.

Elution volume (ml)

µ Moles maltose hydrolysed/min/ml enzyme at 37°C

µ Moles maltose hydrolysed/min/mg protein at 37°C
TABLE I. Purification of acid α-glucosidase from rat liver.

Recovery was calculated on the basis of the total enzyme activity from the proceeding step.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Vol. (ml.)</th>
<th>Enzyme activity (units/ml.)</th>
<th>Protein (mg./ml.)</th>
<th>Specific activity (units/mg.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract. (105,000 g supernatant)</td>
<td>90</td>
<td>2081</td>
<td>17.2</td>
<td>120.9</td>
<td>100</td>
</tr>
<tr>
<td>Acid precipitation (NH₄)₂SO₄ Fractionation (30-60% Satd.)</td>
<td>115</td>
<td>1387</td>
<td>6.2</td>
<td>223.8</td>
<td>85</td>
</tr>
<tr>
<td>Sephadex G-100 Chromatography*</td>
<td>35</td>
<td>385</td>
<td>0.8</td>
<td>469</td>
<td>23</td>
</tr>
</tbody>
</table>

* Enzyme eluted between 40 and 70 ml. (Fig. 13)

'Specific activity is defined as moles maltose hydrolysed/min./mg. protein at 37°C and pH 3.6.'
Fig. 14. Rat liver α-glucosidase activity with varying pH after Sephadex G-100 filtration.

- o-, acid and - - -, neutral α-glucosidase activities.
approximately 4.6, whereas peak II had an optimum at pH 7.2; the latter also contained an acid α-glucosidase component which exhibited an optimum at pH 4.0. These values are similar to those obtained by other workers, that is, 6.0 to 7.0 for neutral α-glucosidases (148,161) and 4.0 to 5.0 for acid α-glucosidases (145, 148,161). Abdullah et al (144) also found three α-glucosidases in rabbit-skeletal muscle which were separated by salt fractionation into a neutral enzyme and a mixture of acid and neutral enzymes.

The peak II fraction was tested for acid and neutral α-glucosidase activity after heat-inactivation at 55°C for 30 min. at pH 4.5; 17.6% of the acid enzyme activity was lost, and 66.7% of the neutral enzyme. Since the rates of heat inactivation of the acid and neutral α-glucosidases were different, the acid α-glucosidase activity of peak II is unlikely to be due to the neutral enzyme functioning under acidic conditions. Hence it would appear that rat liver contains at least two acid α-glucosidases of differing molecular weights. Auricchio et al (160) reported similar findings.

The acid α-glucosidase preparation (peak I) was shown to be heterogeneous by polyacrylamide gel electrophoresis.
One major protein band was apparent together with a second minor band.

Molecular weight determinations on the acid (peak I) and neutral (peak II) enzymes were carried out using Sephadex columns which had been precalibrated with proteins of known molecular weights (308).

The elution volume of peak II (containing neutral and acid α-glucosidases) from a Sephadex G-100 column is indicative of an apparent molecular weight of 27,500 (see Fig. 15(a)). The acid α-glucosidase (peak I) possessed an elution volume from a G-100 column which was outside the range of calibration for accurate assessment of molecular weight. An estimate was, therefore, made using a Sephadex G-200 column which gave a value of 179,900 (see Fig. 15(b)). However, the results obtained by gel filtration were far higher than those obtained by Auricchio et al using sucrose-density gradient sedimentation analysis; these workers obtained values of 76,000-83,000 for rat liver acid α-glucosidase (169). More recent determinations by Jeffrey et al (168) on the acid enzyme indicate a molecular weight within the range 109,000-114,000.

The high molecular weight of the rat liver acid α-glucosidase suggests that the enzyme possesses sub-unit structure and partial confirmation of this has been obtained by treating the enzyme with \( \text{M} \)-urea prior to passing it through a Sephadex G-100 column. Fig. 16 shows that at least seven major protein components or sub-units (I-VII) result from this treatment and using a Sephadex G-100 column, equilibrated with \( \text{M} \)-urea, and precalibrated with proteins of known molecular weight. The apparent molecular weights of these sub-units were shown to be:

<table>
<thead>
<tr>
<th>Peak</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>81,280</td>
</tr>
<tr>
<td>II</td>
<td>60,260</td>
</tr>
<tr>
<td>III</td>
<td>50,500</td>
</tr>
</tbody>
</table>
Fig. 15. Determination of the molecular weights of acid and neutral α-glucosidases by Sephadex G-100 and Sephadex G-200 gel filtration.

Proteins used for calibration were: (a), α-chymotrypsin; (b), pepsin; (c), horseradish peroxidase; (d), ovalbumin; (e), haemoglobin; (f), bovine serum albumin; (g), hexokinase; (h), lactate dehydrogenase; (i), α-galactosidase I.
Fig. 16. Sephadex G-100 chromatography of rat liver acid α-glucosidase in...
Peak | Molecular weight
---|---
IV  | 27,000
V   | 23,000
VI  | 20,500
VII | 8,200

These estimates of molecular weights based on gel filtration data are only rough approximations since it is known that the chromatographic behaviour of proteins on Sephadex can be appreciably altered in the presence of urea (309). The subunits (peaks I to VII) produced by dissociation were individually pooled and concentrated by freeze-drying. Each fraction was taken up in deionised water and dialysed against six changes of deionised water at 4°C. The dialysates were again concentrated by freeze-drying and redissolved in 0.1M-sodium acetate buffer, pH 4.8. No acid α-glucosidase activity could be detected in any of the individual fractions and when they were recombined again, no activity was observed. It is supposed that the urea produced some irreversible change in the physical structure.

The location of acid α-glucosidase in subcellular fractions of liver tissue was investigated (see page 37) and the results of differential centrifugation studies are shown in Table II. Acid phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2) activity was used as an enzyme marker for lysosome detection (290) and also as a test of the homogenisation procedure.

The lysosomal fraction contained 38% of the total acid phosphatase recovered which was in good agreement with that of 40% obtained by De Duve et al (290). The high level of acid phosphatase in the soluble supernatant showed that lysosomal rupture had occurred during the initial homogenation. The latter could largely account for the high acid α-glucosidase activity in the supernatant fraction. The lysosomes possessed only 21.5% of total acid α-glucosidase activity. However, the specific activity of the lysosomal enzyme was 5 times greater than that of the enzyme in the soluble supernatant.
TABLE II. Intracellular distribution of acid phosphatase and acid α-glucosidase in rat liver.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Acid Phosphatase</th>
<th>Acid α-Glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enzyme Activity</td>
<td>Specific Activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(units/ml)</td>
<td>(units/mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Recovery (%)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Homogenate</td>
<td>55</td>
<td>3.300</td>
<td>2.408</td>
<td>0.73</td>
</tr>
<tr>
<td>Nuclei</td>
<td>20</td>
<td>2.080</td>
<td>0.826</td>
<td>0.40</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>20</td>
<td>1.080</td>
<td>0.602</td>
<td>0.56</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>20</td>
<td>0.385</td>
<td>2.520</td>
<td>6.54</td>
</tr>
<tr>
<td>Microsomes</td>
<td>20</td>
<td>0.960</td>
<td>0.560</td>
<td>0.58</td>
</tr>
<tr>
<td>Soluble Supernatant</td>
<td>105</td>
<td>0.795</td>
<td>0.322</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Mitochondria were separated from lysosomes by discontinuous density gradient centrifugation (see p. 125).
Some enzyme activity was lost during the separation of mitochondria and lysosomes, which was achieved by the use of a discontinuous sucrose-density gradient. This was due to the fact that interfacial regions, where 'banding' of the fractions occurred, were discarded.

pH optima studies were carried out on the α-glucosidases in the lysosomal, microsomal and soluble supernatant fractions (Fig. 17). The latter fraction possessed both acid (pH 4.0) and neutral (pH 7.0-7.4) activities. In the lysosomes, however, only acid α-glucosidase could be detected and the microsomal fraction possessed mainly neutral activity together with a small amount of the acid enzyme. The subcellular distribution of α-glucosidases agreed with that reported by Lejeune et al (148). Torres and Olavarria (158), however, could detect no neutral α-glucosidase in the microsomal fraction of rat liver; only an acid α-glucosidase, similar to that in the lysosomes, was found; in the case of these experiments the animals were starved for 20 hr. To explain the findings of Lejeune et al (148), Torres and Olavarria (158) suggested that α-amylase acting on its endogenous substrate (particulate glycogen) interfered with the α-glucosidase assay. However, the rats used to obtain the results shown in Fig. 17 were starved for 24 hr prior to experimentation and it seems unlikely that, under these conditions, any endogenous glycogen interfered with the assays. Also, there was an absence of chloride ions in the incubation mixture.

Before further studies on lysosomal acid α-glucosidase were carried out, it was necessary to devise a technique for large-scale preparation of rat liver lysosomes with the lowest possible contamination by mitochondria. Zonal centrifugation was used for this purpose mainly because of the small capacities of commercially available swinging-bucket rotors. The first step in this work was to ensure that a linear sucrose-density gradient was produced and retained in a titanium BKIV zonal rotor before and after a centrifugation run; Fig. 18 shows that this could be achieved.
Fig. 17. α-Glucosidase activity with varying pH after differential centrifugation of rat liver.

- X -, lysosomal fraction; - o -, microsomal fraction;
- △ -, soluble supernatant.
Fig. 18. Rate-zonal centrifugation of a light mitochondrial fraction

- =, sucrose-density gradient; ---o---, specific activity
- - , specific activity of fumarate dehydrogenase.
Rat livers were always subjected to differential centrifugation before using the zonal rotor and in this way a light mitochondrial fraction was obtained and most of the unbroken cells, nuclei and microsomes removed. Table III shows the results of an experiment in which a light mitochondrial fraction of rat liver was subjected to zonal centrifugation at - 45,000 r.p.m. for 3 hr. (isopycnic banding). Acid phosphatase was used as a biochemical marker for lysosomes and glutamate dehydrogenase (L-Glutamate: NAD oxidoreductase; EC 1.4.1.3) for mitochondria.

**TABLE III.** Isopycnic banding of lysosomes and mitochondria.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Acid Phosphatase</th>
<th>Glutamate Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enzyme Activity (units/ml)</td>
<td>Specific Activity (units/mg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enzyme Activity (milli-units/ml)</td>
<td>Specific Activity (milli-units/mg)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>10</td>
<td>1.15</td>
<td>2.06</td>
<td>1.79</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>10</td>
<td>0.55</td>
<td>7.24</td>
<td>13.16</td>
</tr>
</tbody>
</table>

The mitochondria banded between the volumes 510 and 538 ml, and the lysosomes between 533 and 554 ml. Both fractions were found, by biochemical analyses, to be cross-contaminated as well as to sediment at very similar isopycnic positions. Schuel et al (310) had similar difficulties with this technique.

Preparations of lysosomes free from mitochondria have been made in the zonal centrifuge, by Schuel et al (297), on the basis of differences in sedimentation velocity through sucrose gradients. The sedimentation coefficients of lysosomes and mitochondria were found to be 9,400S and 33,300S respectively, assuming that the density of each particle was 1.2g.cm\(^{-3}\) (311). Fig.18 shows the results of a rate-zonal
centrifugation in a linear with volume sucrose-density gradient of a light mitochondrial fraction of rat liver. The main advantage of this technique, apart from large-scale lysosome preparation, is that there is a wide separation of the mitochondrial and lysosomal bands compared with isopycnic centrifugation.

The mitochondria, as indicated by the biochemical marker, glutamate dehydrogenase, sedimented far out into the gradient between the volumes 325 and 500ml. This region of the gradient contained a low level of acid phosphatase and acid α-glucosidase activities.

The lysosomes, as indicated by the biochemical markers of acid phosphatase and acid α-glucosidase, sedimented at a slower rate and at a shorter distance from the starting-sample zone to form two main peaks. One at a volume of 125ml (17% sucrose; 1.0719g cm\(^{-3}\)) and another at 225ml (20.6% sucrose; 1.0881g cm\(^{-3}\)). A third peak is observed at a volume of 525ml (39.3% sucrose; 1.1800g cm\(^{-3}\)) which is almost at the isopycnic banding position for lysosomes.

Electron microscopy of the subcellular particles present in the gradient (shown in Fig.18) served as morphological evidence as to the purity of the fractions collected. Fig.19 shows an electron micrograph of normal intact rat liver and clearly indicates the presence of mitochondria and lysosomes. Glycogen particles are also present and the α- and β-particles of Drochmans (49) can be distinguished.

The zonal fraction 50-100ml (corresponding to the soluble phase of the original homogenate) contains no discernable mitochondria or lysosomes (Fig.20) but appears to consist of debris such as ruptured membranes. 10.4% of the total acid phosphatase activity and 10.5% of the total acid α-glucosidase activity in the gradient remained in the 50-100ml fraction. These observations suggest that excessive lysosomal damage does not take place during homogenisation.

The lysosomal fraction of the gradient (250-275ml) is not cross-contaminated with glycogen particles (Fig.21). This is to be expected since the rats were initially starved for 24 hr. The mean diameter of
Fig. 19. *Electron micrograph of normal intact rat liver.*
Fig. 20. Electron micrograph of the zonal fraction, 50-100 ml.
Fig. 21. Electron micrograph of the zonal fraction, 250-375 ml.
the lysosomes in the electron micrograph is 0.5μ. The fraction is contaminated by microsomal vesicles as was observed by Schuel et al (297). This contamination probably accounts for the relatively low specific activity of acid phosphatase in the lysosomal region in comparison to the high specific activity levels detected in the highly purified lysosomes obtained by Sawant et al (212); even these lysosomal preparations were contaminated (10-15%) by microsomes. Few mitochondria were present in this fraction.

The electron micrographs of the pellet obtained from the mitochondrial region of the gradient (525-550 ml.) are shown in Fig.22. The cristae within the mitochondria appear to be swollen, and the electron density of the mitochondrial matrix is greater than that found in the normal organelles in situ in rat liver (compare with Fig.19). The diameter range of the mitochondria is 0.4-1.0μ and few swollen mitochondria are also present (diameter 0.7-1.03μ). The condensed morphology is characteristic of organelles isolated in sucrose solutions (313). Only a few contaminating microsomes and lysosomes can be seen in the electron micrographs of this mitochondrial fraction.

Rate-zonal centrifugation thus provides a highly reproducible method for separating lysosomes and mitochondria (as shown by biochemical markers and electron microscopy), from a light mitochondrial fraction of rat liver; some cross-contamination does occur, however. In the further studies to be described all lysosomes were prepared by this procedure and fractions between 150-300 ml. (see Fig.18) pooled thus excluding debris at the starting-zone and the majority of the faster sedimenting mitochondria. Table IV shows the percentage of enzyme activities in the 150-300 ml. fraction (Fig.18) recovered from a light mitochondrial fraction that was placed in the zonal rotor and subjected to rate-centrifugation. The results are the means of three independent experiments. 9% of the total glutamate
Fig. 22. Electron micrograph of the zonal fraction, 525-550 ml.
dehydrogenase activity was recovered in the lysosomal preparation and this technique effected a better lysosome-mitochondria separation compared with isopycnic banding (see Table IV).

**TABLE IV.** Percentage of total enzyme activity recovered in the lysosomal (150-300ml; Fig.18) fraction after rate-zonal centrifugation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% specific Activity Recovered (light mitochondrial fraction - 100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Phosphatase</td>
<td>43.1</td>
</tr>
<tr>
<td>Acid α-Glucosidase</td>
<td>40.3</td>
</tr>
<tr>
<td>Glutamate Dehydrogenase</td>
<td>9.1</td>
</tr>
</tbody>
</table>

The 150-300ml fraction (see Fig.18) from a rate-zonal centrifugation was used in a further attempt to purify rat liver acid α-glucosidase. Purified rat liver lysosomes were removed from the gradient by centrifugation at 5,400g (30 min.) after dilution with 8.5% (w/v) sucrose solution to a final sucrose concentration of 12% (0.4M). The lysosomal pellet was resuspended in 0.1M-sodium acetate buffer (pH 4.8), frozen and thawed six times and the membranes removed by centrifugation at 105,000g (1 hr.). The supernatant solution was subjected to acid precipitation, fractionated with solid ammonium sulphate (see p.134) and then applied to a Sephadex G-100 column. Figure 23 shows the elution profile. Only one major protein peak was observed together with one peak of α-glucosidase activity. A summary of the purification is shown in Table V. The four-fold purification is similar to that obtained by starting with liver homogenates (see Table I).

pH Optima studies showed that the α-glucosidase was of the 'acid type' and that there was little, if any, contamination with neutral α-glucosidase (see Fig.24). The enzyme had maximal activity toward maltose at pH 3.6 - 4.0 and toward glycogen at pH 4.5. These values
Fig. 23. Sephadex G-100 chromatography of acid α-glucosidase from rat liver lysosomes.

- o-, protein; - x-, acid α-glucosidase activity; ---Δ---, acid α-glucosidase specific activity.
TABLE V. Purification of acid α-glucosidase from rat liver lysosomes.

Recovery was calculated on the basis of the total enzyme activity from the preceding step.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Vol. (ml)</th>
<th>Enzyme Activity (units/ml) x10^4</th>
<th>Protein (mg/ml)</th>
<th>Specific Activity (units/mg) x10^4</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosomal Extract (105,000g supernatant)</td>
<td>20</td>
<td>1695</td>
<td>3.5</td>
<td>484.3</td>
<td>100</td>
</tr>
<tr>
<td>Acid Precipitation (NH₄)₂SO₄</td>
<td>22</td>
<td>1200</td>
<td>1.0</td>
<td>1200</td>
<td>78</td>
</tr>
<tr>
<td>Fractionation (30-60% saturation)</td>
<td>5</td>
<td>2173</td>
<td>1.5</td>
<td>1449</td>
<td>41</td>
</tr>
<tr>
<td>Sephadex G-100 Chromatography*</td>
<td>24</td>
<td>103</td>
<td>0.05</td>
<td>2160</td>
<td>23</td>
</tr>
</tbody>
</table>

*Enzyme eluted between 67 and 88 ml.

/Specific activity is defined as μmoles maltose hydrolysed/min/mg protein at 37°C and pH 3.6.

are in good agreement with those recently found by Jeffrey et al (168) of pH 3.7 and pH 4.4, respectively, using a highly purified acid α-glucosidase preparation.

In the present study, the enzyme was found to be heterogeneous by polyacrylamide gel electrophoresis with two similar banding patterns to those found with the enzyme from rat liver homogenates (see page 72).

Jeffrey et al (168) have purified acid α-glucosidase from rat liver lysosomes 1300-fold and they also found that their preparation was heterogeneous when examined by polyacrylamide gel electrophoresis. The faint, slower moving band which was observed by these workers was believed
Fig. 24. pH Optima for α-glucosidase from rat liver lysosomes.

- o-, maltose substrate (1.6mg maltose);
- x-, glycogen substrate (3.2mg glycogen).
to be undissociated enzyme, since it possessed enzyme activity, whilst the darker, faster moving band, with no activity, was thought to consist of enzyme subunits which had been produced by the electrophoretic conditions.

A major problem encountered in the purification of acid α-glucosidase from rat liver homogenates (see Table I) or from purified rat liver lysosomes (see Table V) was the stability of the enzyme. Storage of both types of preparations at 0-4°C, for relatively short periods of time, resulted in a very rapid loss of enzyme activity (see Table VI). Jeffrey et al (168) overcame this problem by including 2-mercaptoethanol in the eluting buffer during the gel-filtration stages of purification. This presumably prevents oxidation of protein thiol groups which are at the active site and/or are involved in the association of subunits.

### Table VI. The effect of storage of purified acid α-glucosidase at 0-4°C on activity.

<table>
<thead>
<tr>
<th>Period of storage at 0-4°C (hr.)</th>
<th>% Acid α-Glucosidase Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified from whole rat liver (see Table I)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>77</td>
</tr>
<tr>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>72</td>
<td>27</td>
</tr>
<tr>
<td>96</td>
<td>0</td>
</tr>
</tbody>
</table>
Hormonal control of acid α-glucosidase activity.

In view of the important but poorly understood role of hormones in carbohydrate metabolism, experiments were designed to investigate the possible interaction of hormones with lysosomal acid α-glucosidase and the likelihood that glycogen degradation could be controlled and, hence, Type II glycogenosis be treated.

In preliminary experiments, the effects on acid α-glucosidase of hormones that were known to regulate carbohydrate metabolism (particularly those which influenced blood glucose levels) were investigated.

Intraperitoneal injections of adrenaline, insulin, glucagon, cortisone cortisol and thyroxine into male rats yielded the results shown in Fig. 25. It can be seen that cortisone acetate injection after 2 hr. gave rise to a 1.7-fold increase in the total activity of liver acid α-glucosidase. Thyroxine also increased the enzyme activity; it was noted that glucagon produced a marked decrease in activity.

As cortisone acetate gave rise to the largest increase, further investigations with this hormone were then undertaken in order to explain the activation effect. In view of the rapid action of cortisone it appeared likely that a direct enzyme-hormone interaction was taking place.

Figures 26A and 26B again show clearly that injection of cortisone acetate into male rats increases the total activity of liver acid α-glucosidase. The stimulatory effect reaches a maximum with a cortisone concentration of 24 mg/kg. body weight and with fixed amounts of the hormone, enzyme activity was highest 2 hr. after injection. In order to see if repeated hormone injection would further raise the level of activity, assays on liver homogenates were made at 2 hr. after a 12 mg/kg. body weight injection and again 2 hr. after a second such injection (Fig. 26C). This produced no further increase in total activity.

The effect of cortisone acetate on rat liver slices was studied to exclude the possibility that the release of endogenous hormones or other compounds from other parts of the body were producing the
Fig. 25. Hormonal effects on acid \(\alpha\)-glucosidase activity in vivo.
8 male rats were used for each experiment.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Activity (molecules maltose hydrolysed/min/mg protein at 37°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.01</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>0.02</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.03</td>
</tr>
<tr>
<td>Glucagon</td>
<td>0.02</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.03</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.02</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Fig. 26. The effect of cortisone acetate on rat liver acid α-glucosidase in vivo.

- o- , cortisone acetate-treated animals;
- - e- - , control animals injected with saline;
6 rats were used for each experimental point.
Fig. 26. The effect of repeated injections (12mg/kg body weight) of cortisone acetate on rat liver acid α-glucosidase activity.

-○-, cortisone acetate-treated animals;
---•---, control animals injected with saline.
6 rats were used for each experimental point.
stimulatory effect. The results (Figs. 27A and 27B) were similar to those obtained in the in vivo experiments. The stimulatory effect reached a maximum with a cortisone acetate concentration of 4mg/200mg (wet weight) of liver slices and with fixed amounts of the hormone (2mg/300mg wet weight of liver slices); the total acid α-glucosidase activity was highest after a 2hr. incubation period. The neutral α-glucosidase activity of the slices, however, was not stimulated by cortisone acetate (Fig. 28).

The possibility that other steroids could activate acid α-glucosidase in rat liver slices was also studied. Figures 29A, B, C and D illustrate experiments with cholesterol, 4-pregnen-21-ol-3,11,20-trione, 4-pregnen-17α,21-ol-3,20-dione and adrenosterone, respectively; none of the hormones had any significant effect on acid α-glucosidase activity.

The observation that acid, but not neutral, α-glucosidase was activated by cortisone suggested that the mechanism of activation might involve the liver lysosomes in view of the known interaction of these organelles with hormones (see p. 63).

With purified rat liver lysosomes were incubated for 1 hr. with cortisone acetate, then ruptured by freezing and thawing, activation of the soluble acid α-glucosidase was observed and this reached a maximum at a hormone concentration of 40μg/mg of soluble lysosomal protein (Fig. 30). On the other hand, incubation of a soluble acid α-glucosidase preparation (30-60% ammonium sulphate fraction) from rat liver with varying concentrations of cortisone acetate resulted in no increased activity. In fact, both in the presence and absence of cortisone acetate, inactivation of the enzyme occurred (Fig. 31). The hypothesis was advanced further and the experimental results illustrated in Fig. 32 suggest that activation is dependent upon the lysosomes being intact. In these experiments, it was shown that cortisone acetate had only a small effect if incubated with ruptured lysosomes; preincubation of the intact organelles was necessary in order to effect stimulation of the enzyme. Figure 32 also shows that cortisone acetate cannot
Fig. 27. The effect of cortisone acetate on acid α-glucosidase activity in rat liver slices.

—o—, cortisone acetate-treated liver slices; 
---o---, control liver slices.
Each point represents the arithmetical mean of 10 determinations.
Fig. 28. The effect of cortisone acetate on total neutral α-glucosidase activity in rat liver slices.

— . — , cortisone acetate-treated liver slices;

— — , control liver slices.

Each point represents the arithmetical mean of 10 determinations.

Liver slices incubated with 2 mg. cortisone acetate/300 mg. of liver slice.
Fig. 25A. The effect of cholesterol on acid \(\alpha\)-glucosidase activity in rat liver slices.

I, liver slices (200mg) incubated with 2mg cholesterol over a period of 4 hr.

II, liver slices (200mg) incubated for 2 hr. with varying amounts of cholesterol.

- o --, steroid-treated liver slices;
- ---o---, control liver slices.

Each point represents the arithmetical mean of 8 determinations.
Fig. 29B. The effect of 4-pregnen-21-ol-3,11,20-trione on acid a-glucosidase in rat liver slices.

I, liver slices (200mg) incubated with 2mg of steroid over a period of 4 hr.

II, liver slices (200mg) incubated for 2hr. with varying amounts of steroid.

- o -, steroid-treated liver slices;
---o---, control liver slices.

Each point represents the arithmetical mean of 8 determinations.

4-pregnen-21-ol-3,11,20-trione

[Chemical structure diagram]
Fig. 29C. The effect of 4-pregnen-17α,21-ol-3,20-dione on acid α-glucosidase activity in rat liver slices.

I, liver slices (200mg) incubated with 2mg of steroid over a period of 4 hr.

II, liver slices (200mg) incubated for 2 hr. with varying amounts of steroid.

- o - , steroid-treated liver slices;
--- e ---, control liver slices.

Each point represents the arithmetical mean of 8 determinations.
Fig. 29D. The effect of adrenosterone on acid α-glucosidase activity in rat liver slices.

I, liver slices (200mg) incubated with 2mg of steroid over a period of 4 hr.

II, liver slices (200mg) incubated for 2 hr. with varying amounts of steroid.

- o -, steroid-treated liver slices;
---o---, control liver slices.

Each point represents the arithmetical mean of 8 determinations.
Fig. 30. The effect of cortisone acetate on soluble acid α-glucosidase activity in purified rat liver lysosomes.

Concentration of cortisone acetate (µg/mg soluble lysosomal protein)
Fig. 31. The effect of cortisone acetate on a soluble acid α-glucosidase preparation (30-60% ammonium sulphate fraction) from rat liver after incubation at 37°C.

A. ---, incubation with cortisone acetate;
   ----, incubation in the absence of cortisone acetate;
   concentration of cortisone acetate, 0.6 mg./ml; protein concentration, 14 mg./ml.

B. Incubation time of 30 min.; protein concentration 14 mg./ml.

![Graph A]

![Graph B]
Fig. 32. The effect of cortisone acetate on soluble acid \( \alpha \)-glucosidase activity in purified rat liver lysosomes.

I. Intact lysosomes incubated with cortisone acetate (40\( \mu \)g/mg soluble lysosomal protein) for 1 hr. at 37\(^\circ\)C, then ruptured, centrifuged and acid \( \alpha \)-glucosidase assayed in the soluble fraction, ---\( \circ \)--;

II. Lysosomes incubated with buffer alone, ruptured in the presence of cortisone acetate (40\( \mu \)g/mg soluble lysosomal protein) and then treated as in I; ---\( x \)--;

III. Intact lysosomes incubated with buffer only, then ruptured, centrifuged and assayed, ---\( o \)--.
stabilise the acid α-glucosidase activity. However, to explain the inactivation of the enzyme within the lysosomes by cortisone acetate concentrations in excess of 40μg/mg soluble lysosomal protein (see Fig.30), there are two main possibilities. The first possibility is that there is direct inhibition of the enzyme and the second is that excess steroid produces lysis of the lysosomal membrane with the consequent release of enzyme from the organelle. This latter possibility could be attributed to excess cortisone acetate replacing the endogenous cholesterol molecules within the lysosomal membrane in such a way as to alter the structure and physical properties of the membrane, as postulated by De Duve et al (299).

Jeffrey et al (168) have found K⁺, Na⁺, Li⁺, NH₄⁺, Ca²⁺ and Mg²⁺ ions to be activators of lysosomal acid α-glucosidase activity; K⁺ ions being the strongest activator. This suggested that cortisone acetate might activate the enzyme indirectly by increasing the permeability of the lysosomal membrane to inorganic ions (K⁺, Na⁺, Ca²⁺ and Mg²⁺ ions) from the incubation medium which then activate the enzyme. In order to account for the lack of activation of a soluble acid α-glucosidase preparation (30-60% ammonium sulphate fraction) and activation of the enzyme in intact lysosomes by cortisone, it must also be postulated that within the organelle, the enzyme is in a particular conformation which allows rapid reaction with inorganic ions. Table VII summarises the results obtained by incubating intact lysosomes with and without cortisone acetate in the presence and absence of inorganic ions (Krebs-Ringer bicarbonate solution). The K⁺, Na⁺, Ca²⁺ and Mg²⁺ ions in the Krebs-Ringer bicarbonate solution had little effect on cortisone activation of the enzyme.

It seemed possible that the stimulatory effect produced by cortisone acetate was non-specific and that the hormone would activate other lysosomal enzymes; hence, its reactions with acid phosphatase and β-galactosidase (β-D-Galactoside galactohydrolase; EC 3.2.1.23) were
Table VII. The effect of incubating intact rat liver lysosomes with and without cortisone acetate in the presence and absence of K⁺, Na⁺, Ca²⁺ and Mg²⁺ ions.

The 105,000g soluble lysosomal supernatant solution was assayed for acid α-glucosidase activity.

<table>
<thead>
<tr>
<th>Incubation medium (1 ml)</th>
<th>Acid α-glucosidase. (Specific activity after 1 hr. incubation). ( \times 10^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0.25M-sucrose solution</td>
<td>240</td>
</tr>
<tr>
<td>2. 0.25M-sucrose solution cortisone acetate (40 µg/mg lysosomal protein)</td>
<td>330</td>
</tr>
<tr>
<td>3. Krebs-Ringer bicarbonate -0.25M-sucrose solution</td>
<td>180</td>
</tr>
<tr>
<td>4. Krebs-Ringer bicarbonate -0.25M-sucrose solution cortisone acetate (40 µg/mg lysosomal protein)</td>
<td>340</td>
</tr>
</tbody>
</table>

Specific activity is moles maltose hydrolysed/min/mg protein at 37°C and pH 3.6. Similar results were obtained in 3 independent experiments.
examined. Here again, intact lysosomes were incubated with varying concentrations of cortisone acetate for 1 hr., then ruptured and the assays carried out. In both cases, inhibition rather than activation was observed (Fig. 33). Weissman (314) and Lewis et al. (302) also reported that steroids did not affect acid phosphatase activity in lysosomes. It would appear, therefore, that acid α-glucosidase activation by cortisone acetate is a fairly specific reaction.

Attempts were again made to try and activate acid α-glucosidase (this time in intact lysosomes) with steroids structurally related to cortisone. Hence, the effects of cholesterol, 4-pregnen-21-ol-3,11,20-trione (11-dehydrocorticosterone) and 4-pregnen-17α,21-ol-3,20-dione (11-deoxycorticosterone) on the enzyme were tested by incubating these compounds with purified rat liver lysosomes for 1 hr. at 37°C. This investigation (Figs. 34A, B and C) showed that none of these steroids were capable of activating the enzyme. Although the number of compounds tested was strictly limited, it does appear that relatively minor changes to the structure of cortisone removes its activating property. In particular, the results suggest that the following groups may be involved in the reaction:

(i), the -C-CH₂OH attached at C-17;

(ii), the 17α-hydroxyl group;

(iii), the carbonyl group at C-11.

The possible role of the lysosomal membrane in the cortisone acetate activation phenomenon was examined by incubating purified rat liver lysosomes with ¹⁴C-4-cortisone acetate and washing the organelles five times with buffer to remove the excess labelled steroid. The lysosomes were then ruptured and the membrane washings
Fig. 33. Effect of cortisone acetate on rat liver lysosomal hydrolases.

Intact purified lysosomes were incubated for 1 hr. in the presence and absence of cortisone acetate.

(a) Acid Phosphatase

(b) β-Galactosidase
Fig. 34A  Effect of cholesterol on rat liver lysosomal α-glucosidase activity.

1 hr. lysosomal incubation with varying concentrations of cholesterol at 37°C prior to rupture and then enzyme assay.
Fig. 34B Effect of 4-pregnén-21-ol-3,11,20-trione on rat liver lysosomal acid α-glucosidase activity.

1 hr. lysosomal incubation with varying concentrations of steroid at 37°C prior to rupture and then enzyme assay.

![Chemical Structure](image)

Concentration of steroid (µg/mg soluble lysosomal protein)
Fig. 34 C Effect of 4-pregnen-17α,21-ol-3,20-dione on rat liver lysosomal acid α-glucosidase activity.

1 hr. lysosomal incubation with varying concentrations of steroid at 37°C prior to rupture and then enzyme assay;
added to the soluble lysosomal supernatant. The radioactivity in the soluble and membrane-bound fractions was finally determined. Table VII expresses the results of these experiments and shows that approximately one-third of the total recovered radioactivity was in the soluble fraction from the lysosomes, whereas the remainder was associated with the membrane fraction.

Experiments were then carried out to see whether cortisone acetate activated the membrane bound acid α-glucosidase, as well as the soluble enzyme.

Table VIII Incubation of purified rat liver lysosomes, for 1 hr. at 37°C with 14C-4-cortisone acetate.

The results are the mean of five experiments.

<table>
<thead>
<tr>
<th>Total Incubated</th>
<th>Total recovered</th>
<th>Washing (1-5) lysosomes incubation volume</th>
<th>Final lysosomal washing</th>
<th>Final membroane washing</th>
<th>Lyso-somal contents</th>
<th>Lyso-somal membranes contents</th>
<th>Lyso-somal membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>230,685</td>
<td>218,666</td>
<td>212,223</td>
<td>0</td>
<td>0</td>
<td>1,411</td>
<td>5,032</td>
<td>6,443</td>
</tr>
</tbody>
</table>
The histogram in Fig. 35 represents the results obtained by assaying acid α-glucosidase in the membrane and soluble fractions of purified rat liver lysosomes after incubating the intact organelles with and without cortisone acetate. There is a 100% increase in the total specific activity of the enzyme 1 hr. after incubating the lysosomes with cortisone acetate (40 μg./mg. soluble lysosomal protein). This was not observed with a lower steroid concentration. In these experiments, washing of the lysosomal membranes followed by repeated freezing and thawing ensured that all the lysosomes were ruptured and that the membranes were washed free of soluble enzyme. Three successive washings were necessary to remove all of the soluble acid α-glucosidase from the membrane fraction. The major increase in activity appeared in the soluble fraction (lysosomal contents plus washings) which showed a 288% increase in comparison with a similar fraction obtained by incubating lysosomes with buffer in the absence of steroid. The total activity of the membrane-bound enzyme remained constant.

It should be noted that other lysosomal enzymes have been reported to be bound in part to the lysosomal membrane and these include aryl-sulphatase A (aryl-sulphate sulphohydrolase; EC 3.1.6.1), an esterase, β-acetylglucosaminidase (β-2-Acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase; EC 3.2.1.30), β-glucosidase (β-D-Glucoside glucohydrolase; EC 3.2.1.21) and acid phosphatase (315).

In an endeavour to understand the details of the interaction between cortisone acetate and acid α-glucosidase, some kinetic studies were carried out. The effects of cholesterol, 4-pregnen-21-ol-3,11,20-trione and 4-pregnen-17α,21-ol-3,20-dione on the rates of hydrolysis of maltose by lysosomal acid α-glucosidase are shown in Figs. 36, 37 and 38, respectively. The steroids were in all cases incubated with intact lysosomes and the soluble enzyme fraction then assayed. None of these steroids increased the rate of maltose hydrolysis; cholesterol actually decreased the rate. The effect of cortisone acetate on the
Fig. 35. Effect of cortisone acetate on soluble and membrane-bound acid α-glucosidase from purified rat liver lysosomes.

In each experiment, Img/ml. total soluble lysosomal protein was used; w₁, w₂, w₃ and w₄ represents washings 1,2,3 and 4, respectively, of the lysosomal membranes.
Fig. 36. **Effect of cholesterol on rat liver lysosomal \( \alpha \)-glucosidase activity.**

Rate of maltose hydrolysis by acid \( \alpha \)-glucosidase, after a 1 hr. incubation at 37°C, in the absence, ---\( \circ \)---, and presence, \( \circ \)-\( \circ \), of 45 \( \mu \)g cholesterol/mg. total lysosomal protein.
Fig. 37. Effect of 4-pregnen-21-ol-3,11,20-trione on rat liver lysosomal acid α-glucosidase activity.

Rate of maltose hydrolysis by acid α-glucosidase, after a 1 hr. incubation at 37°C, in the absence, — — — —, and presence, — o —, of 31μg steroid/mg total lysosomal protein.
Fig. 38. Effect of 4-pregn-17β,21-ol-3,20-dione on rat liver lysosomal acid α-glucosidase activity.

Rate of maltose hydrolysis by acid α-glucosidase, after a 1 hr. incubation at 37°C, in the absence, ---, and presence, --o--, of 29μg steroid/mg total lysosomal protein.
rate of hydrolysis of maltose by both soluble and bound lysosomal enzymes is shown in Fig. 39. At a cortisone acetate concentration of 40μg./mg. total lysosomal protein, the rate of hydrolysis by the soluble acid α-glucosidase was almost doubled, whereas there was a small decrease in the rate in the case of the washed membrane-bound enzyme. At a cortisone concentration of 27μg./mg. total lysosomal protein, the hydrolysis rate was slightly inhibited.

No change in the rate of hydrolysis over a 60 min. period was noted when cortisone acetate was added to a dialysed, 30-60% ammonium sulphate fraction of rat liver (42μg cortisone acetate/mg protein) which was then incubated for 15 min. before assaying for acid α-glucosidase (see Fig. 40).

Lineweaver-Burk plots of soluble acid α-glucosidase activities of cortisone-treated and untreated lysosomes are shown in Fig. 41. The V\textsubscript{max} value of 594.6 x 10\textsuperscript{4} μmoles maltose hydrolysed/min./mg protein at 37°C of the enzyme from the steroid-treated organelles was greater than that obtained from the control lysosomes; 312.8 x 10\textsuperscript{4} μmoles maltose hydrolysed/min./mg protein at 37°C. The K\textsubscript{m} values were the same (11.1mM) for both enzymes, however. This finding suggests that cortisone acetate has no effect on the binding of maltose by the enzyme but only increases its catalytic efficiency. Cox et al (316) obtained similar results when studying cortisol-induction of alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) in HeLa 65 cells. In this case, also, cortisol only affected the V\textsubscript{max} value.

The effect of cortisone acetate on the V\textsubscript{max} value for the hydrolysis of maltose by the enzyme suggested that the steroid might, in some way, change the conformation of the enzyme protein and the possibility that this would be reflected in a change in the pH optimum of the enzyme was, therefore, investigated. Figure 42 shows however, that the pH optimum of 4.4 is not affected by cortisone acetate.
Fig. 39. The effect of cortisone acetate on the rate of hydrolysis of maltose by rat liver lysosomal acid α-glucosidase.

A, soluble lysosomal enzyme; B, membrane-bound enzyme. Intact lysosomes were incubated in the absence of cortisone acetate, ————, and in the presence of 27μg steroid/mg total lysosomal protein, —○—, and 40μg steroid/mg total lysosomal protein, —×—.
Fig. 40. Effect of cortisone acetate on the rate of maltose hydrolysis by acid α-glucosidase in a 30-60% ammonium sulphate fraction of rat liver.

15 min. pre-incubation in the absence, ---•---, and in the presence, ---○---, of 42 μg cortisone acetate/mg protein.
Fig. 41. Effect of cortisone acetate on $K_m$ and $V_{max}$ values on rat liver soluble lysosomal acid α-glucosidase activity.

A 1 hr. lysosomal incubation period was carried out before assay, --o--, incubation with 40 μg cortisone acetate/mg total lysosomal protein; ---e--- incubation in the absence of cortisone acetate.
Fig. 42. Effect of cortisone acetate on the pH optimum of lysosomal acid α-glucosidase activity.

A 1 hr. lysosomal incubation at 37°C was used in the absence, ---o---, and presence, -o-, of 36 μg cortisone acetate/mg total lysosomal protein.
The mechanism by which glucocorticoids affect some enzymes of glycogen metabolism has been discussed in the Introduction of this thesis (see page 49). Recently it has been postulated that the cell nucleus is the site at which corticoids exert their primary effects. Adrenal corticosteroids stimulate hepatic RNA polymerase activity in the nucleus (316) and RNA synthesis (317), as well as the synthesis of certain enzyme proteins (316); hence it was suggested that the mechanism by which these hormones enhanced gluconeogenesis was via RNA synthesis. Other investigators do not accept the view that the effects of glucocorticoids on RNA metabolism and enzyme synthesis are the primary action of these hormones but suggest that corticosteroids modify gluconeogenic pathways rather than initiate enzyme components of these pathways (319). Gardner and Tomkins (320) have obtained a soluble, cytoplasmic, macromolecular steroid-binding fraction which forms a strong association with adrenal corticosteroids. It is suggested that it may be involved in transporting the steroid into the cell, or from one cell compartment to another (including the nucleus), or from one cell to another. Beato et al (321) found a cortisol-binding glycoprotein fraction or receptor, in the cytosol which was involved in the transport of the hormone into nuclei of rat liver cells. They concluded that the receptor facilitates the interaction of the hormone with the chromosomal protein, giving rise to an increased transcription of rat liver chromatin and hence resulting in an increased template capacity of the chromatin for RNA synthesis. In the case of alkaline phosphatase, Cox et al (316) suggested that cortisol initiated the synthesis of a modifier molecule which activated the enzyme.

Any type of mechanism involving nucleic acid or protein synthesis is unlikely to explain cortisone acetate activation of acid α-glucosidase since the enzyme is not only activated in organised tissues but also in isolated, purified rat liver lysosomes, which have no apparent synthetic capability. It is, of course, conceivable
that different mechanisms exist in vivo and in vitro, but for the purposes of this discussion, only the activation of isolated lysosomes will be considered.

Steroids are known to stabilise (300-302) and also lyse (302) isolated lysosomes, depending on the concentration of steroid used. De Duve et al (299) suggested that steroids exerted their effects by replacing cholesterol molecules at certain sites on the membranes thus modifying the structure of the latter. A popular theory of steroid action maintains that these molecules are inserted into the lipid bilayers of cellular membranes, either replacing or supplementing the endogenous cholesterol (305,314). A similar phenomenon has been observed with ‘artificial lysosomes’ (liposomes). Graham and Green (322) found that there was a direct relationship between binding of cortisol to plasma membranes of rat liver and cholesterol depletion from the membranes. Pak and Gershfeld (323) suggested that bound steroids exert effects by altering the aqueous environment adjacent to the steroid receptor sites; it is known that the binding of cholesterol molecules in membranes depends strongly on the ordering of water molecules around them (324).

Any permeability changes in the lysosomal membrane, which facilitate the entry of inorganic ions, can be ruled out as a possible mechanism for the activation of acid \( \alpha \)-glucosidase by cortisone (cf. Jeffrey et al (172)) since the activating effect can be observed in the absence of inorganic salts. However, there is good evidence that lysosomal membranes are involved in some way because: (1), activation occurs only with intact organelles and (2), \(^{14}\)C-4-cortisone acetate binds mainly to the lysosomal membrane. However, one should not overlook the possible importance of the cortisone acetate which penetrates into the interior of the lysosomes.

Evidence is available that lysosomal enzymes (or at least a proportion of the molecules of each enzyme) are bound to the membrane and that most are easily released (325). Some workers believe that
lysosomal enzymes are bound to differing charged sites of the membrane (326) and others that they are non-specifically adsorbed (327). Verity and Reith (328) have stated that the protein, as well as the lipid, of the lysosomal membrane may be involved in vivo in the binding of some enzymes to the membrane. Also, that the lysosomal membrane may confer stability on the enzyme by protecting its easily oxidisable thiol groups (329).

An acidic lipoprotein has been isolated from renal (330) and hepatic (331) lysosomes and it has been postulated that this material may form an electrostatic complex with acid hydrolases in the lysosomes and may function as an inhibitor of these enzymes in situ.

When intact lysosomes are treated with cortisone acetate it is the soluble lysosomal acid α-glucosidase that is activated (i.e., there is an increase in specific activity and $V_{\text{max}}$ but not $K_m$) whereas both the total and specific activities of the membrane-bound enzyme apparently remains constant. Therefore, cortisone acetate does not appear to function by releasing bound enzyme into the soluble fraction but presumably, in some way, changes the conformation of the enzyme protein. The reason why cortisone acetate, but not cholesterol, 4-pregnen-21-o1-3,11,20-trione and 4-pregnen-17α,21-o1-3,20-dione, activates acid α-glucosidase could be explained by assuming that there are either selective receptor sites on the lysosomal membrane, or that there is a selective membrane permeability mechanism.

The possible mechanisms by which cortisone acetate stimulates acid α-glucosidase in intact rat liver lysosomes can only be discussed on the basis of the limited information which is at present available. In general terms there appear to be two main possibilities. The first is that cortisone acetate passes, by a specific permeability process, through the lysosomal membrane and then combines with α-glucosidase to form a cortisone-enzyme complex, which is more active than the enzyme itself. The lysosomal membrane may keep the enzyme in an 'organised' state and facilitate complex formation and/or, in
some other way, make the enzyme easily accessible to the steroid. Another possibility is that the cortisone removes an enzyme inhibitor, e.g., an acidic lysosomal lipoprotein (see above). The second is that cortisone acetate may act indirectly and release some other material (perhaps lipid or protein) from the membrane and this then activates the enzyme or removes an inhibitor. The lysosomal membrane could function in the same way as described in the first theory.

The results obtained with rat liver acid α-glucosidase suggest that it may be advantageous to use cortisone therapy in the case of patients with Type II glycogen storage disease. It has been shown that cortisone acetate activates human, as well as rat liver, acid α-glucosidase (Fig. 43).

In relation to the generalised deposition of glycogen in the tissues which occurs in Type II patients, experiments were performed to see if cortisone activation of acid α-glucosidase would lower the level of intralysosomal glycogen. Rats were injected with either saline or with cortisone acetate and the livers removed 2 hr. later. Light mitochondrial fractions were prepared and the glycogen contents of these estimated (Table IX). Cortisone treatment did not appear to lower lysosomal glycogen level. However, this could well be due to the fact that over the 2 hr. period the increased degradative potential produced by cortisone was balanced by further uptake of cytoplasmic glycogen by the organelles. This does not invalidate possible steroid therapy for glycogenesis Type II as, over a substantial period of time, activation of acid α-glucosidase could still result in a general depletion of liver glycogen.

However, the exact cause of the disease is not known and, in particular, whether there is a complete absence of enzyme protein or another primary defect such as the failure of the cell to produce a sub-unit. If such is the case, then cortisone treatment is not likely to be helpful. If some acid α-glucosidase is present, however,
Fig. 43. Effect of incubation, for 1 hr. at 37°C, with different concentrations of cortisone acetate on acid α-glucosidase activity of human liver tissue.
Table IX. Effect of cortisone acetate on intralysosomal glycogen.

Rats were injected with and without 24mg cortisone acetate/kg. body weight and killed 2 hr. later.

<table>
<thead>
<tr>
<th>Rats</th>
<th>µg Glycogen</th>
<th>µg glycogen/g wet liver weight</th>
<th>mean µg glycogen/g wet liver weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>52.2</td>
<td>14.50</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>104.4</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>122.2</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>111.0</td>
<td>19.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>85.8</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>102.6</td>
<td>19.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>92.3</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>59.7</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>24mg./kg. body wt. injection.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>121.2</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>149.2</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>141.7</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>121.2</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>156.7</td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>79.3</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>139.8</td>
<td>21.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>111.9</td>
<td>22.4</td>
<td></td>
</tr>
</tbody>
</table>
cortisone treatment might be beneficial because of the increased catalytic efficiency of the enzyme which is produced by the steroid. If Type II disease results from a failure of the cell to produce any enzyme, it may be possible, in the future, to introduce exogenous α-glucosidase into the tissues. In this connection, the recent studies of Hems et al (261) and Gregoriadis and Ryman (332) suggest that liposomes containing hydrolytic enzymes might be inserted into the liver.
PROCEDURE

Equipment: Analytical grade chemicals were used whenever possible, other chemicals were of the best available purity.

Glass distilled, deionized water was used for preparation of solutions.

Animals: Male Wistar-strain rats, weighing between 150 and 200g, were used both for the studies in vivo and in vitro. The animals were fed on a commercial stock diet ad libitum unless otherwise stated, and were killed as needed for various studies.

Determination of glucose: This was based on the method of Fiske and Subbarow (333) using glucose oxidase with the modification by Cuthbertson (334).

**Preparation of glucose oxidase reagent.** Trypsin (100mg) was dissolved in 2M-HCl (50ml) and diluted to 900ml with water. The pH was adjusted to 7.0 and the solution then diluted to 1 litre. Glucose oxidase (6000IU) was added and the pH again checked and adjusted to 7.0 if necessary.

**EXPERIMENTAL**

Glucose oxidase (0.5mg, Boehringer 13434 Difco), horseradish peroxidase (7.5mg, Boehringer 13582 NCAB) and p-dianisidine dihydrochloride (25mg) were dissolved in the tris-glyceral buffer (250ml) and then stored at 4°C; the reagent was stable for a month.

**Method:** To the sample (1ml) containing glucose in 6ml stoppered glass tubes was added the glucose oxidase reagent (2ml). The solutions were thoroughly mixed and incubated at 37°C for 1 hr. The digest was acidified by adding 1M-sulphuric acid (2ml) and then allowed to stand for 1 hr. to reach room temperature. Absorbance of the resulting pink color was measured at 540 nm.

Standards containing 0-50µg of glucose were used to construct a calibration curve for the glucose oxidase reagent. The addition of cortisone acetate to the digest had no effect on the sensitivity of the reagent.
General Methods

Materials: Analar grade chemicals were used whenever possible; other chemicals were of the best available purity. Glass distilled, deionised water was used for preparation of solutions.

Animals: Male Wistar-strain rats, weighing between 150 and 200g, were used both for the studies in vivo and in vitro. The animals were fed on a commercial stock diet ad lib, unless otherwise stated, and were killed as needed by cervical fracture.

Determination of glucose: This was based on the method of Fleming and Pegler (333) using glucose oxidase with the modification by Catley (334).

Preparation of glucose oxidase reagent: Tris (61g) was dissolved in 5N-HCl (85ml) and diluted to 900ml with water. The pH was adjusted to 7.0 and the solution then diluted to 1 litre. Glycerol (660ml) was added and the pH again checked and adjusted to 7.0 if necessary.

Glucose oxidase (75mg, Boehringer 15424 EGAC), horseradish peroxidase (7.5mg, Boehringer 15302 EPAB) and o-dianisidine dihydrochloride (25mg) were dissolved in the Tris-glycerol buffer (250ml) and when stored at 4°C the reagent was stable for a month.

Method: To the sample (1ml) containing glucose in 6ml stoppered glass tubes was added the glucose oxidase reagent (2ml). The solutions were thoroughly mixed and incubated at 37°C for 1 hr. The digest was acidified by adding 18N-sulphuric acid (2ml) and then allowed to stand for 1 hr. to reach room temperature. Absorbance of the resulting pink colour was measured at 540 nm.

Standards containing 0-50μg of glucose were used to construct a calibration curve for the glucose oxidase reagent. The addition of cortisone acetate to the digest had no effect on the sensitivity of the reagent.
(1). α-Glucosidase assay.

The method was based on that used by Auticchio and Bruni (167) and in all the cases only initial rates of substrate hydrolysis were measured.

The acid α-glucosidase enzyme fraction (0.1 ml.) was added to a mixture of sodium acetate buffer (24 μmole, pH 3.6) and maltose (5.8 μmole), preheated to 37°C, in a total volume of 0.4 ml. Incubation was carried out at 37°C for 15 min. and the reaction was stopped by addition of 0.16M-ZnSO₄ (0.1 ml.) and 0.36M-NaOH (0.1 ml.) solutions. The resulting protein precipitate was removed by centrifugation at 1500 g (5 min.) and a portion of the supernatant solution (0.4 ml.) assayed for glucose by the glucose oxidase method.

Neutral α-glucosidase assay was achieved by the same method with the exception that imidazole-HCl buffer (24 μmole, pH 7.5) replaced sodium acetate.

A unit of enzyme activity is defined as μmole maltose hydrolysed/min./ml. enzyme solution at 37°C.

Specific activities are expressed as μmole maltose hydrolysed/min./mg. of protein 37°C.

(2). Preparation of α-glucosidases and ammonium sulphate fractionation:

Rats were starved for 24 hr. before being killed. The livers were homogenized in an MSB homogenizer (3 min. maximum r.p.m.) with a solution (1:1, w/v) of 1mM-EDTA-25mM-NaCl, adjusted to pH 6.7 with 2N-NaOH (165). The homogenate was frozen and thawed six times and centrifuged for 1 hr. at 100,000g. The supernatant was acidified to pH 4.2 by adding N-CH₃-COOH, with stirring, and centrifuged at 12,000g (20 min.). This was followed by immediate neutralisation of the supernatant with aqueous N-NH₂OH and then centrifugation at 12,000g (20 min.). The clear supernatant was fractionated with solid ammonium sulphate, corresponding to degrees of saturation of 0-20%, 20-40%, 40-60% and 60-80%, and the precipitated proteins removed by
135

centrifugation at 25,000g (30 min.) 10 min. after the ammonium sulphate addition. The precipitates were dissolved in 1mM-EDTA-25mM-NaCl (pH 6.7, 10-15 ml.) solution and centrifuged at 10,000g (20 min.), then dialysed for 12 hr. against several changes of EDTA-NaCl solution. The dialysed preparation (4 ml; 50-100 mg. protein) was applied to a Sephadex G-100 column (2.5 x 40 cm.), prepared as described by Andrews (308) and this was eluted with 0.1M-sodium acetate buffer, pH 4.8, at a flow rate of 15 ml./hr; 5 ml. fractions were collected. Pooled fractions were concentrated by freeze drying and then redissolved in water (5 ml.) and dialysed against 0.1M-sodium acetate buffer, pH 4.8, for 12 hr.

All preparative stages were performed at 0-4°C.

(3). pH optima studies.

In these studies the following buffers were used in the enzyme incubation volume:-

ph 3.2-6.2, citrate-phosphate buffer (24 μmole)
ph 6.4-7.8, imidazole-HCl buffer (24 μmole)

(4). Heat inactivation studies.

The freeze-dried 90-130 ml. fraction (see Fig.13) obtained by Sephadex G-100 chromatography was dissolved in water (5 ml.) and dialysed for 12 hr. against 0.1M-sodium acetate buffer, pH 4.8.

Aliquots of the enzyme solution (1 ml.) were subjected to heat inactivation at 55°C for periods up to 30 min. (162) and then rapidly cooled in ice. A non-heated enzyme preparation served as a control.

Acid and neutral α-glucosidase activities were assayed and the liberated glucose determined by the glucose oxidase method.

(5). Disc electrophoresis.

Electrophoresis on polyacrylamide columns was carried out by the method of Steward et al (337) with a Shandon apparatus. The reservoir buffer (tris-glycine, pH 8.3) was prepared as described by Davis (338). The enzyme preparations (100 μg protein) in 4% (w/v) sucrose solution
(0.2ml.) were applied to the polyacrylamide columns and electrophoresis was carried out for 90 min. at 100v and 3 mA/tube.

(6). **Molecular weight determination by Sephadex-gel filtration.**

Sephadex G-100 and G-200 columns (2.5 x 40 cm.) were prepared as described by Andrews (308) and calibrated, at 0-4°C, by determination of the elution volumes of a number of proteins (100 mg, in 4 ml.) of known molecular weight; using 0.1M-sodium acetate, pH 4.8, for elution at a flow rate of 15 ml./hr. Fractions were collected and elution volumes were found by absorbance measurements at 280 nm. The results were expressed in terms of $R_{av}$ values (i.e. elution volume of bovine serum albumin/elution volume of the protein).

(7). **Subcellular fractionation of rat liver.**

The method employed was essentially that of Schneider and Hogeboom (296). Rats were starved for 24 hr. before being killed. The livers were removed and washed with ice-cold 0.25M-sucrose-1mM-EDTA solution (pH 7.0), blotted on filter paper, and then the liver (10g) was cut into pieces, which were homogenized in 5 vols (w/v) of fresh, ice-cold 0.25M-EDTA solution by six passes of a Potter-Elvehjem type homogenizer with a diameter clearance of 0.48mm. between the Teflon pestle and the sides of the tube. The homogenate was filtered through four layers of muslin and then fractionated on an MSE Superspeed 50 centrifuge by differential centrifugation at 0-4°C, as follows:-
HOMogenate centrifuged for 10 min. at 650 g.

NUCLEAR FRACTION

SUPERNATANT centrifuged for 10 min. at 22,500 g.

SUPERNATANT centrifuged for 30 min. at 100,000 g.

LIgHT MITOCHErDRIAL FRACTION layered on a sucrose-density gradient (1.17-1.23 g/cm³) centrifuged for 3 hr. at 100,000 g.

SOLUBLE SUPERNATANT

MICROSOMAL FRACTION

1.17-

heavy mitochondrial fraction

1.23-

lysosomal fraction

Each sediment was resuspended in the same medium (5 ml.) and recentrifuged at the speed used in isolating the particular cell fraction. This was repeated. The washings were added to the corresponding supernatant solutions.

The preparation of the discontinuous sucrose-density gradient was achieved by carefully pipetting three layers of sucrose solutions into a 6 ml. centrifuge tube: from bottom to top: 48.6% sucrose (w/w, 1.23 g cm⁻³; 1.5 ml.), 43.1% sucrose (w/w, 1.20 g cm⁻³; 1.5 ml.) and 37.4% (w/w, 1.17 g cm⁻³; 1.5 ml.). The light mitochondrial fraction in 0.25 M-sucrose-1 mM EDTA (1.5 ml.) was carefully layered on to the top of the gradient. Separation of the dark-brown lysosomal fraction from the buff-coloured mitochondrial fraction proved to be the most
Fig. 44. Schematic diagram showing fluid line connections with the B-XIV titanium zonal rotor.
whilst the rotor was rotating at 2,500 r.p.m. This was immediately followed by 60% (w/w, 1.2955 g cm$^{-3}$) sucrose solution (50 ml; the 'cushion' fluid), pre-cooled to 0°C, which resulted in the 17% sucrose emerging through line E and indicated that the rotor was full. The temperature for all subsequent operations was kept at 0-4°C. Lines B and E were then clamped and 0.25M-sucrose-1mM-EDTA (-10 ml; 1.0349 g cm$^{-3}$, pH 7.0, 0°C) containing the sample applied with a 20 ml syringe at a flow rate of 2.5 ml/min. to the rotor through line E with line C open. The flow rate was found by measuring the exudate from line C. Air was not allowed to enter line C so that the gradient would not be disturbed by air bubbles. Line B was clamped and the 20 ml syringe replaced by a 50 ml syringe containing light mineral oil with B open, the light oil was pumped into the rotor at a flow rate of 2.5 ml/min. and this ensured the sample zone was moved from the core of the rotor into a region of higher gravitational field. On completion, lines B, C, D and E were clamped, the rotor feed head assembly removed and the rotor capped. After centrifuging the rotor was decelerated to 2,500 r.p.m. before unloading.

(ii) Unloading the rotor: The feed assembly was replaced and the fluid lines B, D and E opened. Sucrose solution (60% w/w) was pumped into the rotor, through line D, at a flow rate of 15 ml/min. The gradient containing the separated sample material was displaced through the rotor core and line B and fractions collected (5 ml.) which were kept surrounded by ice.

To test the efficiency of the gradient in the zonal rotor, refractive index measurements on an Abbey refractometer were made on samples of the thoroughly mixed fractions collected before and after a zonal run. By calibrating the refractometer with sucrose solutions of known % (w/w) composition, the gradient was assessed. The density of the sucrose solution from % (w/w) composition can be found from tables (340).
Zonal centrifugation of rat liver lysosomes.

A light mitochondrial fraction was prepared from rat liver (20g), from animals which had been starved for 24 hr. prior to killing, (see Experiment 7). This fraction was then resuspended in ice-cold sucrose solution (8.5% w/w) to give a final volume of 10 ml., and applied to the zonal centrifuge following Experiment 9.

One of two types of centrifugation was then carried out: either (a), isopycnic banding by centrifuging at 45,000 r.p.m. for 3 hr. or (b), rate-zonal centrifugation by spinning at 11,000 r.p.m. for 4 min. In this latter method, the acceleration (3.75 min.) and deceleration (4.24 min.) times were ignored.

Fractions (25 ml.) were collected from the zonal rotor and the % sucrose composition of each determined by refractive index measurements. The lysosomes and mitochondria were separated from the gradient by centrifugation at 54,000g for 30 min. after dilution with 8.5% sucrose solution to a final sucrose concentration of 12% (0.4M) (341). The pellets were resuspended in ice-cold 25mM-NaCl-1mM-EDTA, (pH 6.7, 1 ml.), frozen and thawed three times and the organelle membranes removed by centrifugation at 100,000g for 1 hr. The supernatant solutions were assayed for acid phosphatase, acid α-glucosidase and glutamate dehydrogenase activities.

Glutamate dehydrogenase assay.

This was carried out with a Boehringer diagnostic kit (15995 TGAD).

Enzyme activity is defined as the amount of enzyme which catalyses the formation 1 μmole of α-oxoglutarate/min./mg. protein at 25°.

Electron microscopy. A rate-zonal centrifugation was carried out on a light mitochondrial fraction of a liver from a starved rat as described in Experiment (10). Pellets from the following collected fractions (see Fig.18) were examined with the electron microscope:
Liver tissue from a rat on a normal diet was also examined.

The samples, in plastic centrifuge tubes, were fixed for 15 min. in 3.25% glutaraldehyde buffered with 0.1M-phosphate buffer at pH 7.3 and then 1mm³ pieces cut from the bottoms of the tubes. The samples were postfixed in 2% osmium tetroxide containing 0.1M-phosphate buffer at pH 7.3 for 1 hr. at 0-4°C (297) and then dehydrated, at the same temperature, through a graded series of ethanol/water mixtures (20, 40, 60, 80 and 90%) and, finally, two changes of absolute ethanol (5 min. immersion in each). This was followed by two treatments (15 min. each) with propylene oxide and the samples were then covered with fresh propylene oxide (2 ml.) plus working solution (2 ml.) (see p.122) and left for 1 hr. The mixture was decanted off and fresh working solution (2 ml.) added and left 6 hr. for infiltration to take place. Blocks were cast with fresh working solution in gelatin capsules and left overnight at 60°C in a vacuum dessicator, to prevent air bubble formation during polymerisation.

The blocks were trimmed and ultra-thin sections were cut with glass knives on a Leitz ultramicrotome (342). The sections were picked up on copper grids, 200 mesh size, coated with formvar. (The coating of the grids was achieved by placing a drop of 1% formvar in chloroform solution on to the surface of sterile water contained in a dish, (9 x 6 in.) in a dust-free environment. A thin film formed on to which was placed the copper grids. The grids and film were picked up on a microscope slide and allowed to dry in a dust-free atmosphere.)

The sections were then subjected to the following staining procedure by inverting the grids on to globules of the stain on a sheet of parafilm:-

(a), 1% uranyl acetate in 50% ethanol for 10 min. (343), then thoroughly washed in sterile water and allowed to dry completely;
(b), lead citrate for 10 min. (344), and
(c), thoroughly washed in sterile water and allowed to dry.
Finally, the stained sections were examined with a Metropolitan-
Vickers EM6 electron microscope.

(13). Hormone injection studies.
Male rats weighing between 150 and 200 g. were injected intra-
peritoneally with the following hormones in saline (1 ml.):-
(i), adrenaline (1mg./kg. body wt.);
(ii), insulin (0.1iu/kg. body wt.);
(iii), glucagon (200ug/kg. body wt.);
(iv), thyroxine (60ug/kg. body wt.);
(v), cortisone acetate (12mg./kg. body wt.). See Experiment (14)
Control animals were injected with 1ml. saline. 8 rats were
used with each hormone.

After the injection (2 hr.), the animals were killed, the livers
immediately excised and each liver independently homogenized in a
1:1(w/v) 1mM-EDTA-25mM-NaCl (pH 6.7) solution in a MSE homogenizer
(3 min., maximum r.p.m.). The homogenate was frozen and thawed six
times using an acetone/dry ice mixture and then centrifuged for 1 hr.
at 100,000g. Acid α-glucosidase activity was determined in the
supernatant solution.

Owing to the relative insolubility of steroids in aqueous media,
injections are given in the form of fine suspensions which are often
difficult to prepare. The best possible results were obtained by
dissolving in propylene glycol (0.5 ml.) at 50°C and then adding
Krebs-Ringer bicarbonate solution or saline solution (100 ml.) (345).
In this procedure, the final concentration of the organic phase in the
medium should not exceed 0.5% (346). In this present study, control
media contained the same volume of propylene glycol as in the test
media containing the steroid.
Liver slice studies.

Liver slices were prepared as described by Deutsch (347).

The slices (200-300 mg. wet weight) were placed in stoppered glass tubes containing Krebs-Ringer bicarbonate solution (10 ml.) with and without added cortisone acetate. The tubes were placed in a shaking incubator at 37°.

After incubation, the slices were removed from the incubation medium, thoroughly washed in ice-cold Krebs-Ringer bicarbonate solution and dried on hard filter paper. The slices were individually homogenized in 1mM-EDTA-25mM-NaCl, pH 6.7 (2 ml.), in a glass-Teflon Potter-Elvehjem homogenizer, frozen and thawed three times in acetone-dry ice and finally centrifuged at 100,000g for 1 hr. (0-4°). Enzyme assays were carried out on the supernatant fractions.

Effect of steroids on purified lysosomes.

The procedure for purifying rat liver lysosomes was based upon Experiments (9) and (10) using a rate-zonal centrifugation. The 150-300 ml. fractions collected from the zonal rotor were pooled and thoroughly mixed, and the sucrose-gradient solution, containing the lysosomal suspension, diluted with ice-cold, 8.5% (w/w) sucrose solution to a final concentration of 12% (w/w) sucrose. Aliquots (35 ml.) were pipetted out and centrifuged at 54,000g (30 min.) at 0-4° (341). The supernatants were poured off and each lysosomal pellet was suspended, by stirring in Krebs-Ringer bicarbonate solution (1 ml.) (345) containing 0.25M-sucrose (85.6 mg. of solid sucrose added/ml. of Krebs-Ringer bicarbonate solution) (348) with and without the steroid. The lysosomes were then incubated at 37° with slow shaking for 1 hr.

After the incubation, the treated lysosomes were removed from the medium by centrifugation at 22,500g (20 min., 0-4°) and twice washed with ice-cold Krebs-Ringer bicarbonate solution containing 0.25M-sucrose and then again centrifuged at 22,500g (20 min., 0-4°).
Ice-cold 1mM-EDTA-25mM-NaCl, pH 6.7 (1 ml.), was added to each pellet which was frozen and thawed three times, using acetone-dry ice, and the organelle membranes removed by centrifugation at 100,000g for 1 hr. at 0-4°C. The supernatant was assayed for enzyme activity.

Determination of the total amount of protein in the lysosomal pellets was carried out by dissolving lysosomal pellets in 1mM-EDTA-25mM-NaCl, pH 6.7 (5 ml.) containing 0.1% Triton X-100 in order to solubilize all the protein. Undissolved material was removed by centrifugation at 100,000g for 1 hr. The supernatant was assayed for protein by a modification of the Folin method (335).

(17). β-Galactosidase assay.

β-Galactosidase was assayed by addition of enzyme solution (0.4 ml.) to a mixture of McIlvaine buffer, pH 5.5 (0.3 ml.) and 30mM-p-nitrophenyl β-D-galactoside (0.3 ml.) preheated to 30°C (349). This temperature was maintained for 15 min. and the reaction was then stopped by the addition of 0.1M-Na2CO3 solution (5 ml.). The release of p-nitrophenol was measured at 405nm.

The enzyme activity is expressed as mmole p-nitrophenyl β-D-galactoside hydrolysed/min./mg. protein at 30°C.

(18). Preparation of lysosomal membranes.

Purified rat liver lysosomes were treated with Krebs-Ringer bicarbonate-0.2M-sucrose (1 ml.) containing cortisone acetate, as described in Experiment (16). Control lysosomes were treated with buffer only.

The lysosomes were frozen and thawed three times in 1mM-EDTA-25mM-NaCl, pH 6.7 (1 ml.) and the membranes removed by centrifugation at 100,000g for 1 hr. The supernatant was decanted and kept frozen at -60°C in an acetone-dry ice bath. The lysosomal membranes were then washed, with vigorous stirring, four times with 1mM-EDTA-25mM-NaCl, pH 6.7 (1 ml.); each washing being followed by freezing and
thawing (three times) and 100,000g centrifugation for 1 hr. The washings were kept frozen at -60°C until required. The freezing and thawing stages were included to ensure total lysosomal rupture.

The membranes were vigorously stirred with 1mM-EDTA-25mM-NaCl, so as to produce a very fine suspension in a final volume of 1 ml. This was carried out in a graduated glass tube.

All operations were performed at 0-4°C.

The lysosomal contents, washings and membranes were assayed for acid α-glucosidase activity. During the 15 min. incubation period in the assay, care was taken to ensure that sufficient shaking of the reaction tubes maintained the lysosomal membranes as a fine suspension.

(19). The effect of 14C-4-cortisone acetate on purified rat liver lysosomes.

14C-4-cortisone acetate was suspended in Krebs-Ringer bicarbonate solution containing 0.25M-sucrose. The supernatant (1 ml.) gave a count of ca. 200,000 cpm.

Purified rat liver lysosomes were incubated with Krebs-Ringer bicarbonate solution containing 0.25M-sucrose and 14C-4-cortisone acetate (1 ml.) in a shaking incubator at 37°C for 1 hr. The lysosomes were removed from the medium by 22,500g centrifugation (20 min.) and washed five times with Krebs-Ringer bicarbonate-0.25M-sucrose solution (1 ml.) with 22,500g centrifugation (20 min.) between each washing.

The lysosomes were then separated into membrane and a soluble fraction as in Experiment (13). The membrane washings were pooled with the soluble fraction.

All the fractions, excluding the lysosomal membranes, were evaporated to dryness under reduced pressure, at room temperature, extracted with benzene/ethanol (90:10, v/v) solution (0.2 ml.), separately spotted on Whatman No.3 paper (4cm² pieces) and then dried thoroughly under an infra-red lamp. The membranes were directly transferred to the paper. Three washings of the flasks and centrifuge
tubes used in the experiment with benzene/ethanol solution (0.2 ml.)
ensured that all the $^{14}$C-4-cortisone acetate was recovered.

The papers carrying the radioactivity were counted to 1% error
with a Beckman LS 100 scintillation counter, using a toluene
scintillant (1 litre of toluene containing 5g/litre PPO and 0.3g/
litre POPOP),
quench corrections were applied to the results obtained.

With this technique, 95% of the $^{14}$C-4-cortisone acetate was
recovered.

(20). Effect of cortisone acetate on a human liver biopsy specimen.

A needle biopsy specimen (5mg, wet weight) was divided into
five, approximately equal, sections which were suspended in Krebs-
Ringer bicarbonate solutions (1 ml.) containing various concentrations
of cortisone acetate. Incubations were carried out at 37° for 1 hr.
The sections were then thoroughly washed with ice-cold, Krebs-Ringer
solution, and dried by touching with hard filter paper. After
freezing and thawing in sodium acetate buffer, pH 3.6 (24µmoles,
0.1 ml.) six times, the tissue suspension were centrifuged at 100,000g
for 1 hr. at 0-4° and the supernatants taken for enzyme assays.

The enzyme solution (0.1 ml.) was added to sodium acetate buffer
(24 µmoles, pH 3.6), 0.5µM-[U-$^{14}$C] maltose (0.2 ml., 0.025µCi) and
0.2µM-D-glucose preheated to 37° in a total volume of 0.5 ml. (245).
Incubation was carried out at 37° for 40 min. and the reaction was
stopped by the addition of propan-2-ol (1 ml.). The resulting protein
precipitate was removed by centrifugation at 1500g (5 min.) and the
supernatant solution fractionated by descending chromatography on
Whatman No.3. paper with ethyl acetate-acetic acid-formic acid-water
(18:3:1:4, by vol.) solvent. The [$^{14}$C]-glucose liberated by the acid
α-glucosidase was determined by counting the radioactivity on appro-
priate 1cm paper strips from the chromatograms with a Beckman LS 100
scintillation counter using a toluene scintillant.
Enzyme activity is expressed as percentage of maltose hydrolysed in 40 min. at 37°C.

(21). **Effect of cortisone acetate on the intralysosomal glycogen content.**

Rats, weighing between 150 and 200g, were injected intraperitoneally either with saline (1 ml.) or 24 mg/cortisone acetate in saline/kg. body weight (1 ml.) and then allowed to feed ad lib. 2hr. after the injection, the animals were killed, the livers immediately excised and a light mitochondrial fraction prepared from each liver as in Experiment (7).

The washed light mitochondrial fractions were boiled in 30% potassium hydroxide solution (3 ml.) in tapered centrifuge tubes, which were placed in a water bath, for 30 min. (34). Loosely fitting glass-stoppers were used to close the mouths of the tubes and the contents of the tubes were occasionally stirred with a glass rod to facilitate dissolution. After 30 min. the layers of fat that separated out on the surfaces of the alkali solutions were removed with a pipette.

The tubes were then cooled in crushed ice and 1.5 volumes of 95% ethanol were added to precipitate the polysaccharide. The precipitates were centrifuged off at 3,000g (10 min.) at 0-4°C and then taken up in water (1 ml.). Purification of the glycogens was effected by three further alcohol precipitations in the presence of a trace of ammonium acetate using the same tubes. The final glycogen pellet was taken up in water (1 ml.) and dialysed overnight against running tap water to remove any co-precipitated mono- and di-saccharides. Finally, the glycogen was reprecipitated with alcohol and taken up in water.

The glycogen content of each solution was determined by the phenol-sulphuric acid method (350) using purified rat liver glycogen as a standard. The results were expressed as μg glycogen/g. wet liver.

8 rats were used as controls and 8 rats for cortisone treatment.


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MATERIALS AND METHODS

All buffer solutions were prepared with reagent grade chemicals in distilled water. The pH of solutions was adjusted with 0.1 N NaOH or 0.1 N HCl. The pH of the enzyme assay was 7.8 and the pH of the substrate assay was 6.0. The enzyme solution was assayed at 37°C. The pH optimum was determined at 37°C using a pH-meter (Radiometer, Copenhagen, Denmark).

Acid phosphatase assay. The reaction mixture contained 0.25 M sodium 4-glycero-phosphate (0.1 ml), 0.125 M sodium acetate buffer, pH 6.0 (0.2 ml) and enzyme fraction (0.1 ml). After incubation for 30 min at 37°C, an equal volume of 10% (w/v) trichloroacetic acid was added, and a sample of the precipitated solution was assayed for P by the method of Fiske & Subbarow (1925). The activity was calculated as µmol of phosphate formed/min at 37°C per mg of protein.

Glutamate dehydrogenase assay. Glutamate dehydrogenase was assayed using a spectrophotometric method (1960 TGAD).

Animals. Male Wistar rats, weighing between 160 and 200 g, were used both in the studies to determine the liver enzyme and tissue culture studies. The animals were fed on a commercial stock diet ad lib. and were killed as needed by cervical fracture.
Studies on Type II Glycogenosis

EFFECTS OF CORTISONE DERIVATIVES ON ACID α-GLUCOSIDASE

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(Received 7 October 1970)

Cortisone causes a marked increase in the activity of liver acid α-glucosidase 2h after injection into male Wistar rats. Studies on rat liver tissue slices, isolated lysosomes and cultured skin fibroblasts have demonstrated similar elevations of acid α-glucosidase activity after incubation with cortisone. Cortisone-treated human liver tissue, obtained by needle biopsy, also shows an increase in acid α-glucosidase activity. Neutral α-glucosidase activity was not stimulated by cortisone in vivo or in liver slices.

All but one of the various types of glycogen storage diseases are characterized by specific enzyme lesions (Brown & Brown, 1968). In type II glycogenosis lysosomal acid α-glucosidase is not detectable and the absence of this activity is believed to result in tissue glycogen accumulation (Hers, 1963; Brown, Brown & Jeffrey, 1970). In the present study the effect of cortisone and cortisol (hydrocortisone) on acid α-glucosidase activity in rats has been examined in relation to possible treatment of cases of type II disease.

MATERIALS AND METHODS

Materials. A.R.-grade chemicals were used whenever possible; other chemicals were of the best available purity. Glucose oxidase (15424 EGAC) and horseradish peroxidase (15302 EPAB) were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K.; o-dianisidine dichloride and cortisone acetate were purchased from British Drug Houses Ltd., Poole, Dorset, U.K.; cortisol (hydrocortisone) sodium succinate was obtained from Glaxo Ltd., Greenford, Middx., U.K.

Difco tissue culture media (Waymouth 752/1 MB, Eagle’s minimal medium) and antibiotics, and bovine serum (T. C. desiccated), were obtained from Baird and Tatlock Ltd., London W.1, U.K.; cortisol (hydrocortisone) sodium succinate was obtained from Glaxo Ltd., Greenford, Middx., U.K.

[U-14C]Maltose was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Acid α-glucosidase assay method I. Sodium acetate (24 mM, pH 3.6), maltose (5.8 μmol) and enzyme fraction (0.1 ml, 100 000 g supernatant) in a total volume of 0.4 ml were incubated together at 37°C for 15 min and the reaction was stopped by addition of 0.18 M ZnSO4 (0.1 ml) and 0.36 M NaOH (0.1 ml) solutions. The protein precipitate was removed by centrifugation and a portion of the supernatant solution (0.4 ml) assayed by the glucose oxidase method (Fleming & Pegler, 1963; Catley, 1967). Specific activity is defined as μmol of maltose hydrolysed/min per mg of protein at 37°C.

Acid α-glucosidase assay method II (Dancis, Hutzler, Lynfield & Cox, 1969). Sodium acetate (0.024 mM, pH 3.6), 0.4 μM [U-14C]maltose (0.2 ml), 0.025 μCi, 0.2 μM d-glucose and fibroblast suspension (0.1 ml) in a total volume of 0.5 ml (pH 3.6) were incubated at 37°C and the reaction was stopped by the addition of propan-2-ol (1.0 ml). The protein precipitate was removed by centrifugation and the supernatant solution fractionated by descending paper chromatography on Whatman no. 3 paper with ethyl acetate-acetic acid-water-formic acid (18:3:1:4, by vol.) solvent. [14C]Glucose liberated by enzymic hydrolysis was determined by counting the radioactivity on appropriate 1 cm paper strips from the chromatograms with a liquid-scintillation counter. A glucose marker was used to locate the radioactive areas of the chromatogram. Enzyme activity is defined as percentage of maltose hydrolysed in 40 min at 37°C.

Acid phosphatase assay. The reaction mixture contained 0.25 M sodium β-glycerophosphate (0.1 ml), 0.125 M sodium acetate buffer, pH 5.0 (0.2 ml) and enzyme fraction (0.1 ml). After incubation for 30 min at 30°C, an equal volume of 10% (w/v) trichloroacetic acid was added, and a sample of the deproteinized solution was assayed for P, by the method of Fiske & Subbarow (1925). The activity was calculated as μmol of phosphate formed/min at 30°C per mg of protein.

Glutamate dehydrogenase assay. Glutamate dehydrogenase assay was carried out with a Boehringer diagnostic kit (15995 TGAD). Animals. Male Wistar rats, weighing between 150 and 200 g, were used both in the studies in vivo and to provide material for the liver enzyme and tissue-culture studies. The animals were fed on a commercial stock diet ad lib. and were killed as needed by cervical fracture.
Studies in vivo. Cortisone acetate (in saline) was injected intraperitoneally (Hanonne & Feigelson, 1969) and the liver excised immediately after death and homogenized in a 1:1 (w/v) 1 mM-EDTA-25 mM-NaCl (pH 6.7) solution in a Waring Blender. The homogenate was frozen and thawed six times and centrifuged for 1 h at 100000 g. \( \alpha \)-Glucosidase activity was determined by assay method I.

Treatment of liver slices. The method of Deutsch (1936) was used to prepare rat liver slices (wet wt. 260–300 mg each) which were placed in a shaking incubator at 37°C in stoppered tubes containing Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) (10 ml) with and without added cortisone acetate. The slices were individually homogenized in 1 mM-EDTA-25 mM-NaCl, pH 6.7 (2 ml), in a glass–Teflon Potter–Elvehjem homogenizer, frozen and thawed three times and finally centrifuged at 100000 g for 1 h. \( \alpha \)-Glucosidase activity was measured by assay method I.

Treatment of lysosomes. The livers of rats that had been starved for 24 h were excised and a light-mitochondrial fraction was prepared by the method of Schneider & Hogeboom (1960, 1965a, b). Further fractionation was effected with a 650 ml linear-density gradient of 17–55% sucrose and centrifuging for 4 min at 11000 rev./min (excluding acceleration and deceleration times) in a B-XIV titanium zonal rotor (MSE Ltd., London S.W.1, U.K.). Acid phosphatase and glutamate dehydrogenase activities served as markers for the lysosomal and mitochondrial fractions, respectively; the former was eluted from the rotor between 150 and 300 ml. This was diluted with 0.25 M sucrose to a final molarity of 0.4 with respect to sucrose, and the organelles were isolated by centrifugation at 54000 g (30 min) (Aronson & de Duve, 1968). Lysosomal incubations were carried out at 37°C in Krebs–Ringer bicarbonate solution (Krebs & Henseleit, 1932) (1 ml) containing 0.25 M sucrose (35.6 mg of solid sucrose added/ml of Krebs–Ringer bicarbonate solution) (Rosenberg & Janoff, 1968) with and without cortisone acetate and the treated lysosomes were removed by centrifugation at 22500 g (20 min) and then ruptured by freezing and thawing three times in 1 mM-EDTA-25 mM-NaCl, pH 6.7 (1 ml). Organelle membranes were removed by centrifugation at 100000 g for 1 h and the supernatant solution was assayed for acid \( \alpha \)-glucosidase activity by using method I. All preparations were carried out at 0–4°C.

Tissue culture. A small section (1 mm \( \times \) 2 mm) of skin was removed from the shaved abdomen of a rat immediately after death and was sterilized in Ringer solution containing penicillin (1000 units/ml) and streptomycin (0.5 mg/ml). The explants were cultured in Eagle’s minimal medium, supplemented with 10% (w/v) bovine serum, for 7 days at 37°C, treated with 0.5% trypsin, and sub-cultured in 12 cm2 plastic dishes containing Waymouth’s medium, supplemented with 8% (w/v) bovine serum. All media were sterilized by filtration through millipore filters. The cells were allowed to grow to confluence. All media contained penicillin (50 units/ml) and streptomycin (50 \( \mu \) g/ml). Cells were harvested by trypsinization followed by centrifugation or by use of a rubber ‘policeman’ and the acid \( \alpha \)-glucosidase activity of suspensions of fibroblasts in 24 mM-sodium acetate buffer, pH 6.0 (0.1 ml), was measured, by assay method II, after incubation at 37°C with and without cortisol sodium succinate.

Treatment of human liver tissues. A needle biopsy specimen (5 mg) was divided into five approximately equal sections which were suspended in Krebs–Ringer bicarbonate solution (1 ml) containing various concentrations of cortisone acetate. (Individual digests contained approximately equal concentrations of protein.) Incubations were carried out at 37°C for 1 h and assay method II was used for acid \( \alpha \)-glucosidase after tissue maceration.

Protein. Protein was measured by a modification of the Folin method (Lowry, Rosebrough, Farr & Randall, 1951) with crystalline human serum albumin as standard.

RESULTS AND DISCUSSION

Figs. 1(a) and 1(b) show clearly that injection of cortisone acetate into rats increases the concentration of acid \( \alpha \)-glucosidase in the liver. The stimulatory effect reaches a maximum with a cortisone concentration of 24 mg/kg body wt. and with fixed amounts of the hormone enzyme activity is highest 2 h after injection. To see if repeated hormone injection would further increase the enzyme activity, acid \( \alpha \)-glucosidase activity was measured 2 h after a 12 mg/kg body wt. injection and again

![Fig. 1. (a) Effect of intraperitoneal injection of cortisone acetate (12 mg/kg body wt.) into rats on liver acid \( \alpha \)-glucosidase activity after various times. •, Animals injected with saline (1 ml); ○, cortisone acetate (in saline, 1 ml)-treated animals. (b) Effect of different concentrations of cortisone acetate on liver acid \( \alpha \)-glucosidase activity measured 2 h after injection. The livers were excised immediately after death and individually homogenized in 1 vol. (v/w) of 1 mM-EDTA-25 mM-NaCl, pH 6.7, in a Waring Blender. The homogenate was frozen and thawed six times, centrifuged at 100000 g for 1 h and the supernatant was assayed by assay method II for acid \( \alpha \)-glucosidase activity. Six rats were used for each experimental point in (a) and (b). The enzyme activity is expressed in \( \mu \)mol of maltose hydrolysed/min per mg of protein at 37°C and the arithmetical mean and range of the results are shown.]
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Fig. 2. Effect of two equal intraperitoneal injections (12 mg/kg body wt.) of cortisone acetate on rat liver acid α-glucosidase activity. The second injection was given 2 h after the first and the assays were carried out 2 h after the injection. O, Cortisone acetate (in 1 ml of saline)-treated animals; #, animals injected with saline only (1 ml). Six rats were used per experimental point and the results were obtained and expressed in the same way as those shown in Fig. 1.

Fig. 3. (a) Acid α-glucosidase activity in rat liver slices (about 300 mg each) after incubation with cortisone acetate (2 mg) for different times. O, Cortisone-treated liver slices; #, controls. (b) Effect of different concentrations of cortisone acetate (2 h incubations) on acid α-glucosidase in rat liver slices (about 200 mg each). O, Cortisone-treated liver slices; #, controls. Each slice was placed in a stoppered tube (containing 10 ml of Krebs-Ringer bicarbonate solution) on a shaking incubator with or without added cortisone acetate. The slices were individually homogenized in 1 mM-EDTA-25 mM-NaCl, pH 6.7 (2 ml), in a glass-Teflon Potter-Elvehjem homogenizer, frozen and thawed three times and centrifuged for 1 h at 100000g. Acid α-glucosidase activity expressed as μmol of maltose hydrolysed/min per mg of protein was determined by assay method I. The experimental points represent the arithmetical and range mean of ten determinations.

Fig. 4. Effect of different cortisone acetate concentrations on the acid α-glucosidase activity in whole rat liver lysosomes (for extraction procedure see the text). The lysosomes were incubated at 37°C in different concentrations of hormone for 1 h before assay. The acid α-glucosidase activity is expressed as μmol of maltose hydrolysed/min per mg of lysosome protein and was determined on a 100000g supernatant, after freezing and thawing three times in 1 mM-EDTA-25 mM-NaCl, pH 6.7 (1 ml). Experimental points are the mean of three determinations.

Table 1. Effect of cortisol on the acid α-glucosidase activity in rat skin fibroblasts

<table>
<thead>
<tr>
<th>Concentration of cortisol (μg/ml)</th>
<th>Maltose hydrolysed (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>3.9</td>
</tr>
<tr>
<td>10</td>
<td>4.3</td>
</tr>
<tr>
<td>100</td>
<td>15.3</td>
</tr>
<tr>
<td>1000</td>
<td>10.1</td>
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</tbody>
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Rat skin fibroblasts grown to confluence in Waymouth’s medium at 37°C were harvested and then incubated with different concentrations of cortisol sodium succinate for 40 min at 37°C. Acid α-glucosidase activity was measured by assay method II (described in the Materials and Methods section).

Treatment of a dialysed 30–60% saturated ammonium sulphate fraction of acid α-glucosidase from rat liver with cortisol acetate produced no activation.

Neutral α-glucosidase activity did not appear to be stimulated by cortisol in experiments either in vivo or with tissue slices.

A needle-biopsy specimen of normal human liver
behaved similarly to rat liver in that cortisone acetate activated acid α-glucosidase and at higher concentrations produced an inhibition (Fig. 5).

The molecular weight of rat liver lysosomal acid α-glucosidase is approx. 114000 (Jeffrey, Brown & Brown, 1970). Our own (unpublished) gel-filtration experiments confirm that the enzyme has a high molecular weight and a probable subunit structure. Cortisone only appears to be an effective activator when the enzyme is in an ‘organized state’ (i.e. intra-cellular or intra-organelle). In this connexion it is not known whether type II glycogenosis results from a complete absence of the enzyme protein or possibly from an incomplete assembly of a number of subunits. The latter could result from a failure of the cell to synthesize one or more subunits or from the lack of some other factor that is responsible for higher-order structures of the protein.

The authors are indebted to the Medical Research Council for their financial support and to Dr P. Golding for a human liver biopsy specimen.

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