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CARBOHYDRATE METABOLISM IN THE HUMAN FETAL AND NEW-BORN TISSUE, WITH SPECIAL REFERENCE TO GLYCOGEN.

A Thesis submitted by

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ABSTRACT

Glycogen has been extracted from human fetal livers ranging in age from 13 to 26 weeks. The average chain lengths have been determined by α-amylolysis and periodate oxidation and the average exterior chain lengths by β-amylolysis. The structures of liver glycogens from baboon, mouse, rabbit and several mature humans have also been determined. The absorption spectra of some glycogen-iodine complexes have been recorded and also glycogen values have been determined by the reaction with concanavalin A. The development of glycogen in the human fetal liver is discussed in relation to other mammalian species.

The activities of the enzymes glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase), phosphoglucomutase (D-glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase), and phosphoglucoisomerase (D-glucose-6-phosphate ketol isomerase) in the human fetus have been studied.

The liver glycogens from four cases of suspected glycogen storage disease have been isolated and the structures determined as previously described. An electrophoretic and chromatographic study has been made of sugars in the urine of another case, where the patient lacked several intestinal enzymes.

The action of glucamylase and a commercial amyloglucosidase preparation ("Diazyme") on glycogen has been examined. The glucamylase and β-amylase limit dextrins of glycogen have been isolated and their average chain lengths determined.

C O N T E N T S

INTRODUCTION

Although Bernard [1] isolated glycogen from dog's liver more than 100 years ago, no detailed structural analysis was undertaken until the 1920's when Karrer [2] and Haworth and his coworkers [3] investigated the methylation and acetylation of the polysaccharide.

Prior to this it was known that glycogen was a glucose polymer,[4] which could be enzymically degraded to a reducing sugar and a mixture of dextrins by diastase, saliva and pancreatic juice.[5] The exact nature of the linkages between the D-glucose units had no significance until the ring structure of D-glucose and other monosaccharides had been postulated and proved.

In mammalian tissues glycogen occurs in liver,[1] skeletal muscle,[6] placental tissue,[7] surface epithelial cells,[8] intestinal mucosal cells,[8] brain,[9] cardiac muscle,[10] and kidney.[11] Many other organisms contain glycogen, for example yeast,[12] fungi,[13] insects [14] and some bacteria such as *Escherichia coli*.[15] Certain plants such as *Zea mays* also contain a polysaccharide possessing the characteristics of glycogen.[16,17] Although chemically indistinguishable from glycogen, their administration to rabbits, dogs and humans has been found to cause an increase in urinary loss of carbohydrate, although the blood carbohydrate levels remain constant.[18] Evidently they do not possess the same physiological characteristics

as the mammalian glycogens. Sumner and Somers [19] refer to them as "phytoglycogens", but it has been suggested [20] that because of their botanical origin they should be considered as highly branched amylopectins.

Conclusive proof that glycogen was a glucose polymer came initially from Külz and Borntträger,[4] who compared the physical properties of authentic glucose and the sugar obtained from the acid hydrolysis of glycogen, and found them to be identical. Methylation and hydrolysis studies by Karrer [2] and Haworth, Hirst and Webb [3] provided further evidence of the constitution of the polysaccharide. Karrer also noted the similarity in chemical behaviour between glycogen and starch.

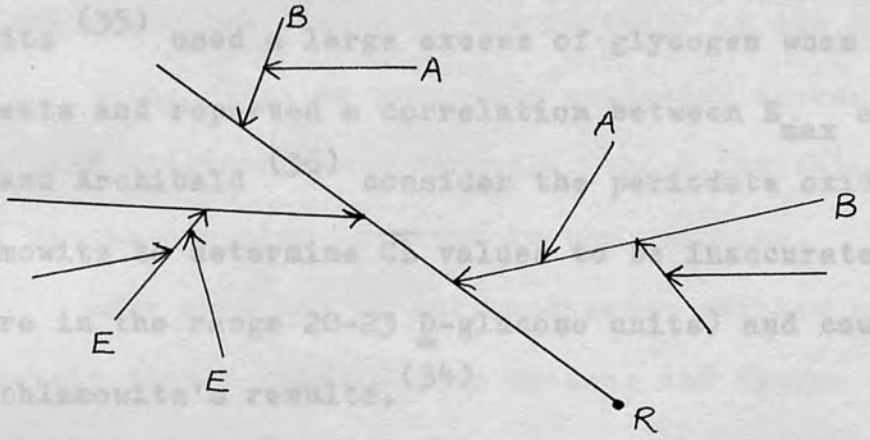
There are several properties common to the three very similar glucosans, i.e. glycogen, and the starch fractions, amylose and amylopectin. All three show a high dextro-rotatory power in aqueous solution, the specific rotation normally being in the region +191 - + 199°.[21,22] The molecular weights [23] are in the region 10^6 - 10^7, these values being average. Many samples of these polysaccharides have been shown to be polydisperse when examined in the ultracentrifuge. Smith and his coworkers have also demonstrated this heterogeneity by electrophoresis on glass-fibre paper.[24]

The infrared spectra are also very similar. In the range 730 - 960 $cm.^{-1}$ the three polysaccharides show peaks at 928±3,

838±3 and 760 ± 2 cm.$^{-1}$ (25) The peak at 838 cm.$^{-1}$ is characteristic of α - linked D - glucopyranose units, and the two other peaks are characteristic of α - D - (1 → 4) - linked glucans.(26)

A reaction which serves to distinguish between the three polysaccharides is the formation of coloured complexes with iodine. Glycogens react with iodine solutions to give a reddish-brown complex, the absorption maximum usually occurring in the region 420 - 490 mμ. The amylopectin-iodine complex is again reddish, having a λ_{max} at 530 - 540 mμ.(27) The colour given by amylose varies with the degree of polymerisation ($\overline{DP}$). Very short chains of amylose have a λ_{max} at about 490 mμ, but these are usually regarded as degradation products, or intermediates in the synthesis of amylose.(28) True amylose has a much higher $\overline{DP}$ and gives a blue colour with iodine. The value of λ_{max} is 600 - 645 mμ (28) It is known that the staining properties of α - D - (1 → 4) - linked glucosans arise from the fact that the D - glucose molecules are arranged in a helix,(29,30) 6 D - glucose units forming one turn of the helix. The iodine molecule or complex iodide ion (31) occupies the space of one turn of the helix. Colouration is not observed until the D - glucose chain is 12 D - glucose units long (28), although some workers consider that 18 D - glucose units are necessary.(32) In the case of

linear molecules such as amylose the absorption maximum of the iodine complex increases as the chain length increases.[28,33] Manners and his coworkers [34] have examined the absorption spectra of glycogen- and amylopectin-iodine complexes, and suggest that the greater colour intensity given by amylopectins, and the difference in the values of λ_{max} between the two compounds arises from the difference in interior chain length, and hence from the difference in B chain length



A chains - chains linked by the reducing group to another chain.

B chains - chains having A chains attached to them.

R = reducing group

E = exterior chains

The average B chain length in amylopectin is 26 and that in glycogen 16. Hence the B chains of amylopectin will contain a large number of helices of a suitable length (18 D - glucose units) to accommodate the iodine molecules, whereas glycogen

will have relatively few. The compactness and consequent impenetrability of the glycogen molecule is suggested as an additional factor which hinders complex formation. No correlation between the chain length of the glycogens and λ_{max} or the extinction coefficient (E_{max}) was noted in aqueous solution, but on addition of 25% or 50% saturated ammonium sulphate [35] an increase in E_{max} and an approximate correlation of average chain length ($\overline{CL}$) with λ_{max} was noted. Schlamowitz [35] used a large excess of glycogen when making these measurements and reported a correlation between E_{max} and $\overline{CL}$. Manners and Archibald [36] consider the periodate oxidation used by Schlamowitz to determine $\overline{CL}$ values to be inaccurate ($\overline{CL}$ values given were in the range 20-23 D-glucose units) and could not repeat Schlamowitz's results.[34]

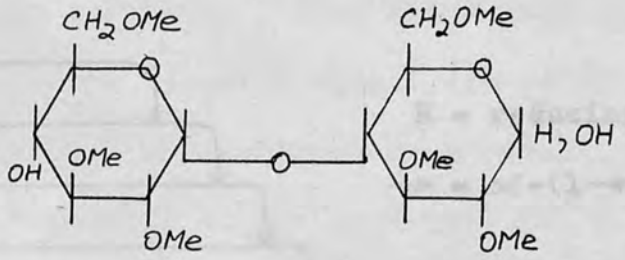
Potentiometric titration data have been studied by Bates, French and Rundle [37], who reported that the affinity for iodine varies directly with $\overline{CL}$ for starches, and that glycogen has a very low iodine affinity. Anderson and Greenwood [38] have improved the technique and report that the iodine-binding power of glycogen is about $^1/_{10}$ that of amylopectins. Only an approximate relationship between iodine-binding power and exterior chain length ($\overline{ECL}$) could be deduced.

Glycogen will form complexes with several proteins [39], but one in particular, concanavalin A, a globulin extracted from

jack-beans,[40] has been used to distinguish glycogens from amylopectin and amylose.[41] Glycogens give a precipitate with solutions of this protein, and the resulting turbidity can be measured spectrophotometrically. Neither amylose nor amylopectin give a precipitate. Since the precipitation occurs most readily with short-chain glycogens and β - limit dextrins, (the molecules remaining after the enzyme β - amylase has removed the outermost chains from glycogen molecules) it was suggested that the reaction causing precipitation occurs in the interior of the glycogen molecule. Methylation or periodate oxidation decreases the reaction with concanavalin A markedly, which indicates that the hydroxyl groups are also involved in the reaction. Manners and Wright [42] have examined 47 polysaccharide samples and report an approximately linear relationship between the degree of branching and the turbidity produced. They have suggested that there are fundamental structural differences between glycogens and amylopectins, as even amylopectins of the same branching characteristics as some glycogens fail to react with concanavalin A.

Studies on the chain lengths and types of linkages present in glycogens were originally based on the classical chemical methods of methylation and periodate oxidation. Haworth and Percival [43] obtained evidence of the α - (1 $\rightarrow$ 4) - linkages in glycogen by isolating a disaccharide from methylated glycogen. On oxidation and methylation this disaccharide gave methyl octa-O-methyl-maltobionate. This proved that glycogen contained α - (1 $\rightarrow$ 4)-linked

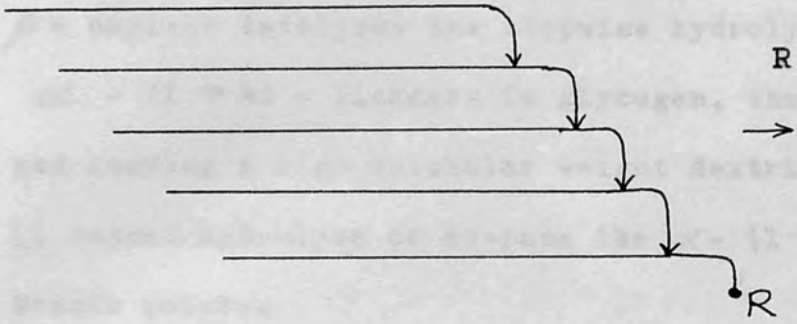
D - glucose units. The structure of the original disaccharide was:-



The same workers isolated 8.9% of methyl tetra-O-methyl-D-glucopyranoside from the methanolysate of methylated glycogen. Since this derivative can be formed only from the non-reducing end-group of a chain of α-(1→4)-linked D - glucose units, they were able to calculate an average value for the chain length (12 D - glucose units) of glycogen. The work of Bell [44-46] supported the findings of Haworth and Percival. Bell also reported the presence of 2,3,6-tri-O-methyl-D-glucose and a di-O-methyl-D-glucose in the acid hydrolysates of methylated rabbit and fish liver glycogens.[47] The di-derivative was later shown to be a mixture of 2,6- and 2,3-di-O-methyl-D - glucoses.[48] The presence of the 2,6- derivative can be interpreted as an indication either of the presence of anomalous α - (1→3) - linkages, or of incomplete methylation of the glycogen molecule.

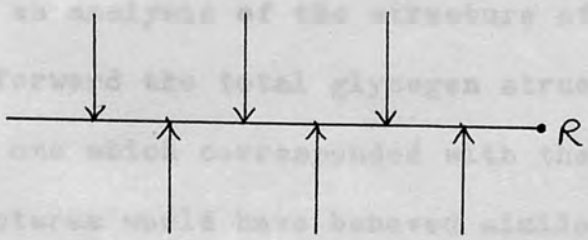
Haworth and Hirst [49] postulated a laminated structure for glycogen in 1937, basing it on the discovery of the high molecular weights of glycogens,[50] and of the presence of 2,3-di-O-methyl-D - glucose in the acid hydrolysates of methylated glycogens,[47]

which arose from the α - (1→6) - linked branch points.

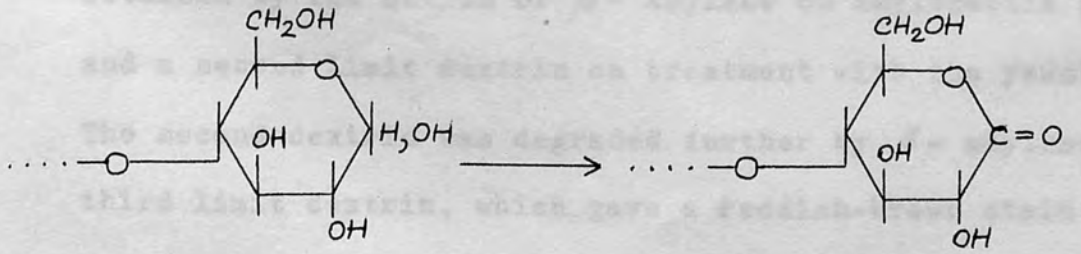


In the laminated structure each chain was considered on average to be 12 D-glucose units long and linked to C-6 of a glucosyl residue in the chain below.

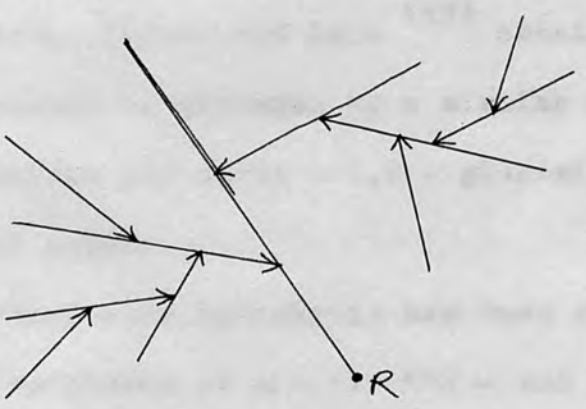
Staudinger [51] put forward a comb-like structure, which allowed for the chemical evidence and also accounted for reports, later disproved, that the glycogen molecule was spherical.



Both formulae explained the lack of reducing power of glycogen previously attributed [52] to the effect of the alkaline extraction medium on the reducing group, in the presence of air.



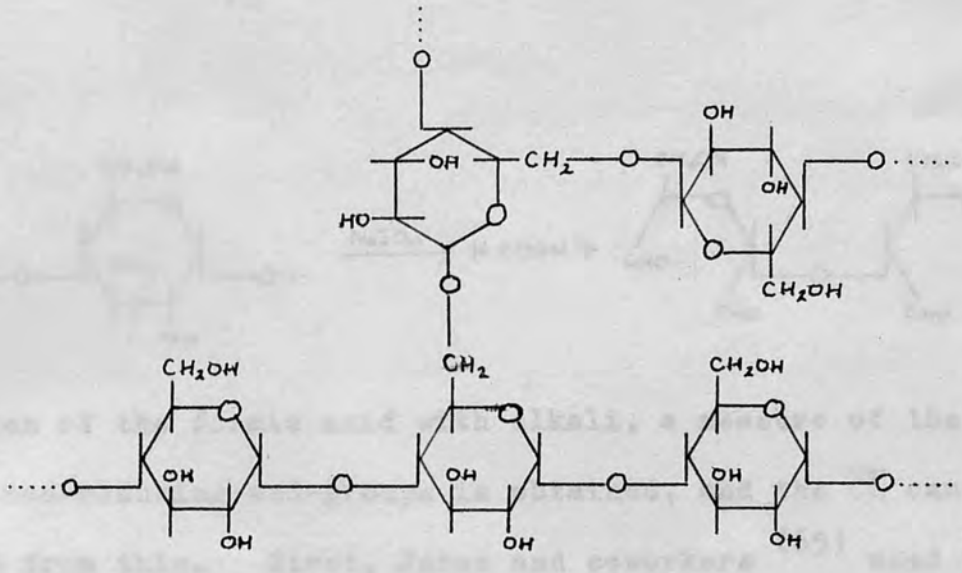
In 1941 Meyer and Fuld [53] postulated the tree-like structure, which is now commonly accepted. They based this upon the fact that β - amylase catalyses the stepwise hydrolysis of alternate α - (1→4) - linkages in glycogen, thus releasing maltose, [54] and leaving a high molecular weight dextrin (β - dextrin) since it cannot hydrolyse or by-pass the α- (1→6) - linkages at the branch points.



From an analysis of the structure of the β- limit dextrin they put forward the total glycogen structure shown above as being the only one which corresponded with their findings. In fact all three structures would have behaved similarly with β- amylase. Meyer and Bernfeld had already established that amylopectin had a randomly branched structure by successive treatments with β- amylase followed by a yeast enzyme preparation. [55] The β - limit dextrin obtained by the action of β- amylase on amylopectin gave D - glucose and a second limit dextrin on treatment with the yeast enzyme. The second dextrin was degraded further by β- amylase to give a third limit dextrin, which gave a reddish-brown stain with iodine.

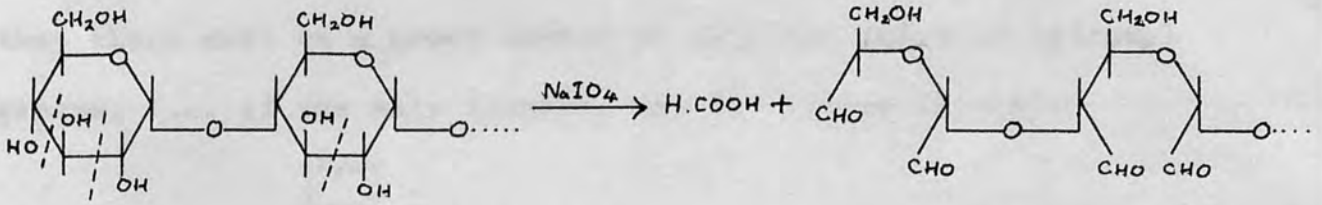
These results were consistent with the randomly branched structure. Structures of the laminated or comb type would have yielded a second dextrin that gave a blue iodine complex and was completely degraded by β- amylase to maltose. It was found that the same yeast enzyme degraded some of the interchain linkages of glycogen β- dextrin [56] and this was taken as proof that the glycogen molecule resembled amylopectin in its branching characteristics. Illingworth, Larner and Cori [57] obtained more rigorous proof of the structure of glycogen by a similar method, substituting phosphorylase and amylo - 1,6 - glucosidase for β- amylase and the yeast enzyme.

Partial acid hydrolysis has been used to support the evidence for the existence of α- (1$\rightarrow$4) - and α- (1$\rightarrow$6) - linkages in glycogen. Wolfrom and his coworkers [58] acetylated the sugars obtained from a partial acid hydrolysate of rabbit liver glycogen and isolated β - isomaltose octa-acetate. Bacon and Bacon isolated isomaltose by partial acid hydrolysis followed by carbon-celite chromatography, [59] and, using the same techniques, Peat, Whelan and Edwards [60] have degraded baker's-yeast glycogen to D - glucose, maltose, isomaltose and panose. Wolfrom and Thompson [61] have isolated isomaltotriose, showing that a small number of chains are attached by α- (1$\rightarrow$6) - glucosidic linkages to adjacent D - glucose residues.



The same workers also isolated 0.001% nigerose from the hydrolysate, suggesting the presence of anomalous α - (1→3) - linkages. This supports the first interpretation of the isolation of 2,6-di-O-methyl-D - glucose by Bell.[48] Smith and coworkers[62] have produced more evidence in support of this by isolating D - glucose after periodate oxidation, reduction and hydrolysis of glycogen. Neither of these more recent findings can be considered conclusive however, as the nigerose could arise from acid-catalysed transglucosylation from maltose[63] and the D - glucose from incomplete periodate oxidation. D - galactose has been isolated from an acid hydrolysate of chick liver glycogen, when the chicks have been fed on a high galactose diet.[64]

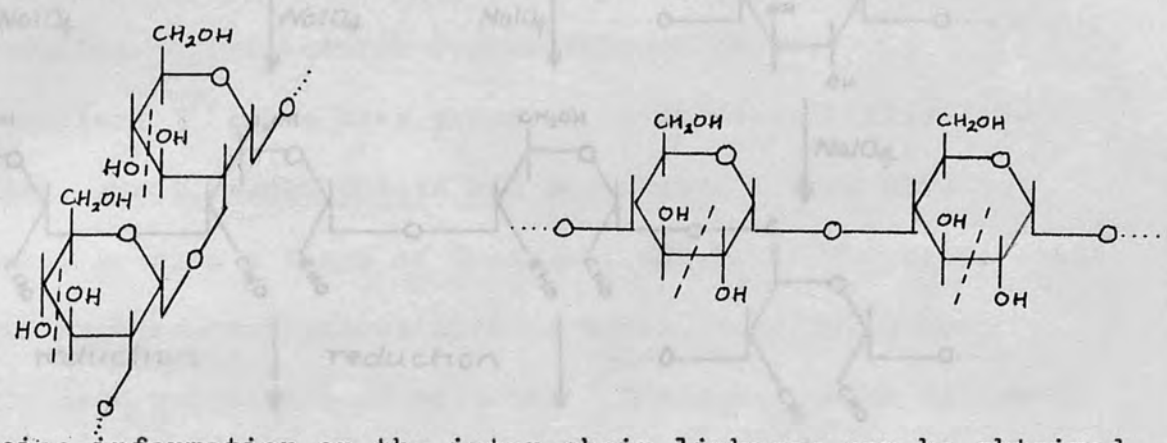
Periodate oxidation has been used as a method for determining the average chain length of glycogen and also for characterisation of the linkages between the D - glucose units. Formic acid is liberated only from the non-reducing end-groups in the glycogen molecule.



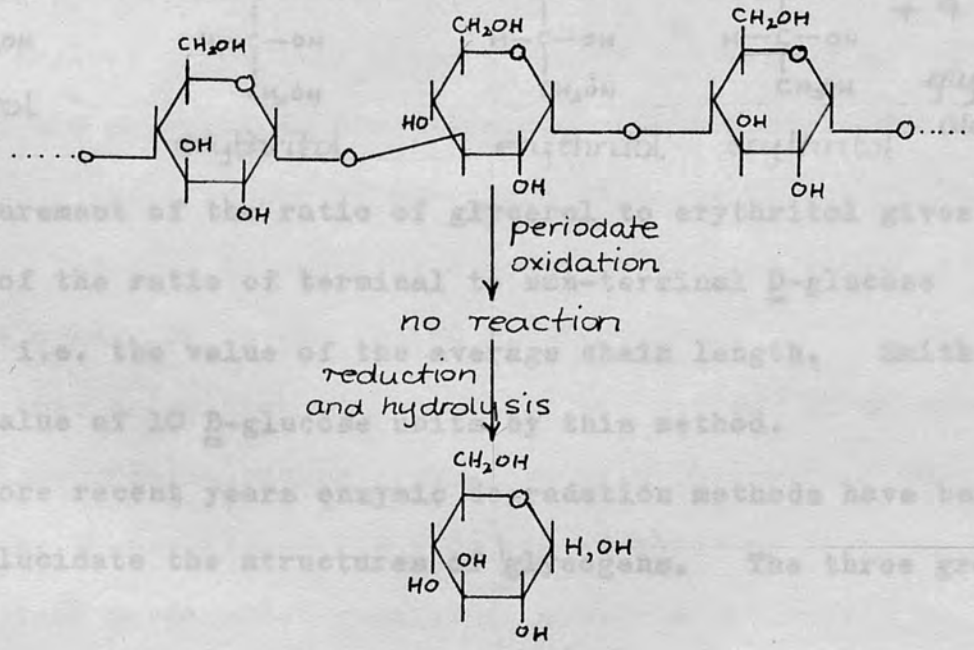
By titration of the formic acid with alkali, a measure of the number of non-reducing end-groups is obtained, and the $\overline{CL}$ can be calculated from this. Hirst, Jones and coworkers [65] used the method to assay a number of glycogens and found the $\overline{CL}$ values corresponded fairly closely with those determined by methylation. Potter and Hassid [66] modified the conditions, substituting sodium metaperiodate for the potassium salt and reducing the temperature and reaction time. This ensured against over-oxidation [67] but unfortunately resulted in incomplete oxidation.[68] Abdel-Akher and Smith [22] increased the oxidation time to 80 hr. and obtained excellent agreement with the results of Halsall, Hirst and Jones.[65] Manners and Archibald [68] suggest 7 - 10 days for complete oxidation using Potter's method and 300 - 400 hr.[69] for the method of Halsall, Hirst and Jones.

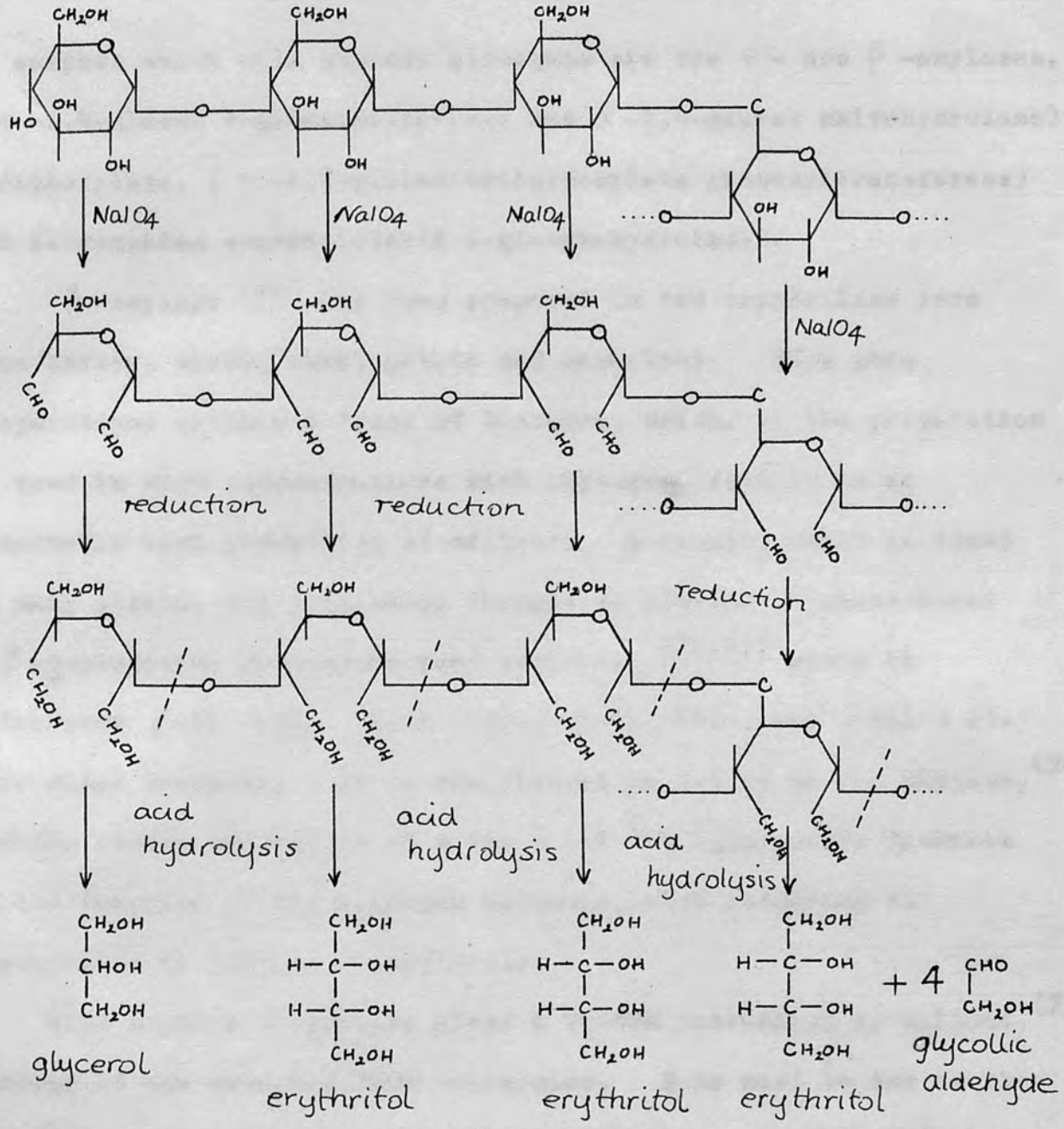
Some information can be obtained from periodate oxidation alone. Formic acid can only be produced if the hydroxyl groups on C-2, C-3 and C-4 are free (see diagram on p. 15). The fact that the amount of formic acid produced is very small indicates that normally one or more of these hydroxyl groups is involved in the chain linkage, and that (1 → 6)- linkages do not form the

main type of linkage. The large consumption of periodate shows that there must be a great number of adjacent pairs of hydroxyl groups, i.e. if the main linkages are (1 → 2) or (1 → 4):



More precise information on the inter-chain linkages can be obtained by acid hydrolysis of the oxidised polysaccharide,[70] preferably after reduction with hydrogen, Raney nickel or sodium borohydride,[62] as the yields of hydrolysed fragments are greatly increased.
The fragments normally consist of polyols, although D-glucose will be obtained if (1 → 3)-linkages are present. Treatment of glycogen in this way results in the following reactions:





Measurement of the ratio of glycerol to erythritol gives an estimate of the ratio of terminal to non-terminal D-glucose residues, i.e. the value of the average chain length. Smith (62) found a value of 10 D-glucose units by this method.

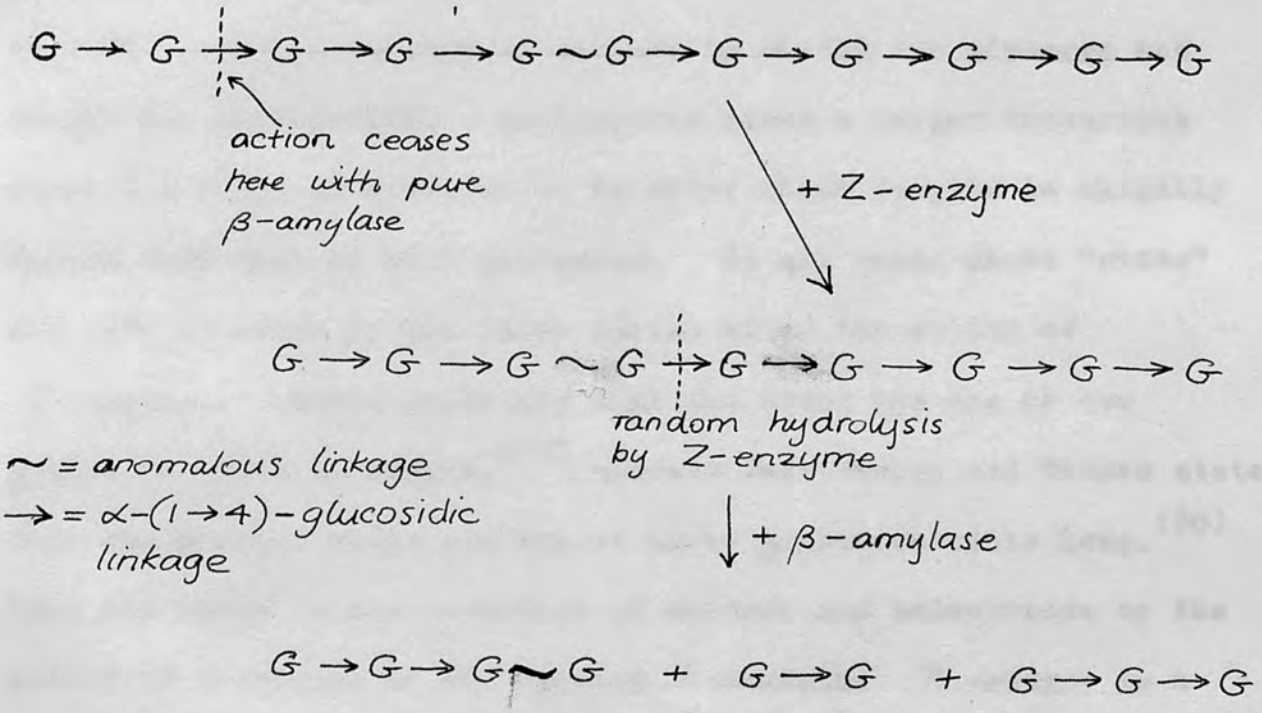
In more recent years enzymic degradation methods have been used to elucidate the structures of glycogens. The three groups

of enzymes which will degrade glycogens are the α - and β -amylases, (α -1,4-glucan 4-glucanohydrolase and α -1,4-glucan maltohydrolase) phosphorylase, (α -1,4-glucan:orthophosphate glucosyltransferase) and debranching enzyme (starch 6-glucanohydrolase).

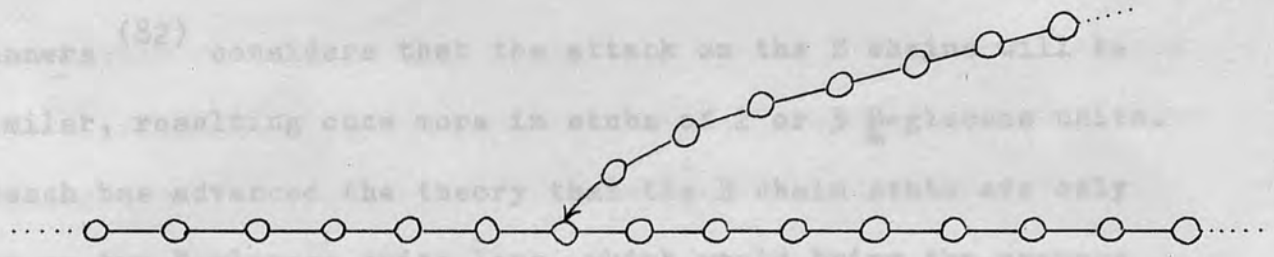
β -amylase [71] has been prepared in the crystalline form from barley, wheat, sweet potato and soya-bean. Even pure preparations contain a trace of Z-enzyme, which, if the preparation is used in high concentrations with glycogen, results in an abnormally high production of maltose. Z-enzyme, which is found in many plants, was originally thought to possess β-glucosidase (β-D-glucoside glucohydrolase) activity, [72,73] since it hydrolysed β-(1$\rightarrow$2), β-(1$\rightarrow$3)-, β-(1$\rightarrow$4)-, and β-(1$\rightarrow$6)-glucosidic linkages. It is now thought to act as an α - amylase, [74] causing random hydrolysis of a few α-(1$\rightarrow$4)-glucosidic linkages in the interior of the glycogen molecule, thus rendering it susceptible to further β -amylolyis.

With amylose β -amylase gives a 70-80% conversion to maltose, [75] instead of the expected 100% conversion. This must be due to the presence of a small number of anomalous linkages in the amylose molecule. Non-terminal phosphate groups have been shown to constitute a barrier to the action of β-amylase. [76] Banks and Greenwood have shown that the anomalies are not due to phosphate groups, since pre-incubation of amylose with phosphatase does not increase the β -amylolysis limit. [77] Addition of Z-enzyme to the β -amylase preparation results in complete conversion to

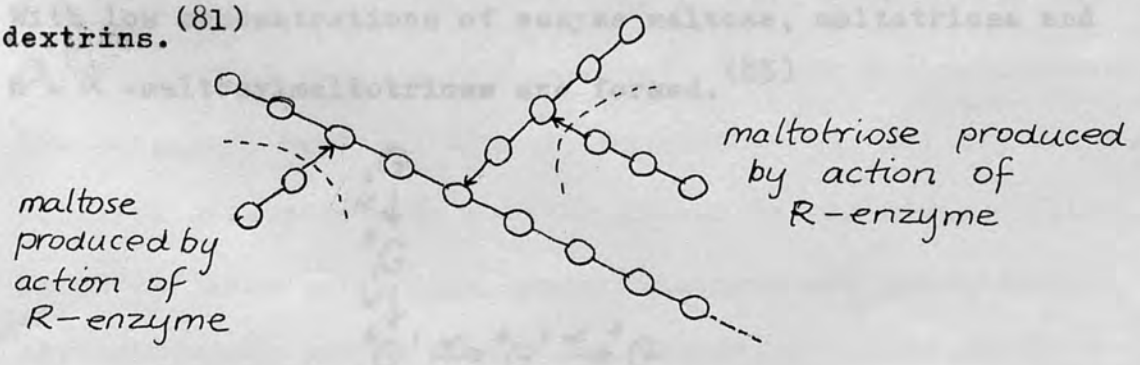
maltose.[75] It was originally thought that Z-enzyme hydrolysed the anomalous linkages,[75] but with the discovery that Z-enzyme activity was an α-amylase activity, the increase in the β-amylolysis limit was seen to be caused by the random hydrolysis of a few of the α-(1 → 4)-linkages. In many cases this would remove the barrier to further β-amylolysis



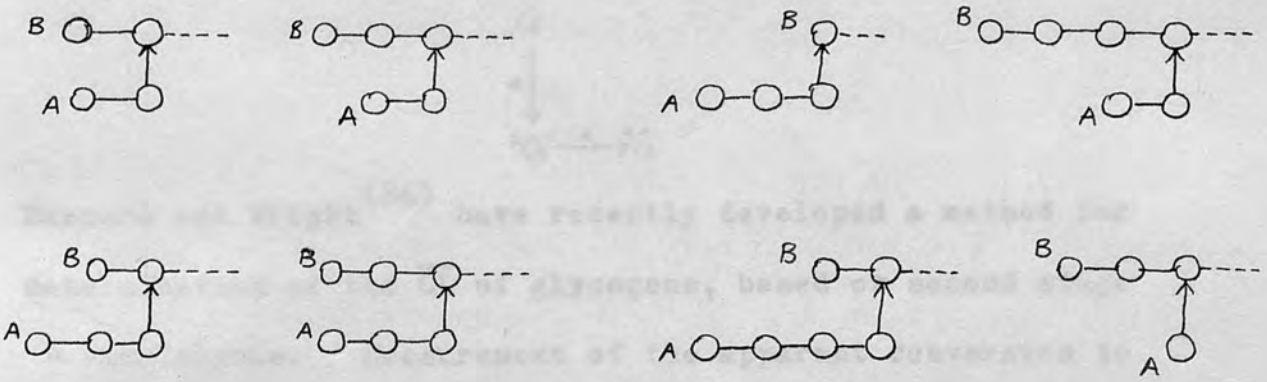
Recently the anomalous linkages have been found to be α-(1 → 6)-glucosidic inter-chain linkages.[78] Treatment of amylose with yeast isoamylase, an enzyme that hydrolyses α-(1 → 6)-glucosidic linkages, followed by β-amylolysis resulted in an increased production of maltose. Manners considers that there are not more than one or two branch points per molecule of amylose



Pure β-amylase cannot bypass the α-(1→6)-linkages in glycogen and amylopectin and hence a high molecular weight dextrin is left. The conversion to maltose is 40-50% for glycogen and 50-60% for amylopectin. Amylopectin gives a larger conversion since its ratio of exterior to interior chain lengths is slightly larger than that of most glycogens. In all cases short "stubs" are left attached to the inner chains after the action of β-amylase. Meyer considers that the stubs are one or two D-glucose units in length,[79] whereas Peat, Whelan and Thomas state that the A chain stubs are two or three D-glucose units long.[80] This was based on the formation of maltose and maltotriose by the action of R-enzyme on amylopectin β-dextrin. R-enzyme is a debranching enzyme which occurs only in plants. It will hydrolyse the α-(1→6)-glucosidic linkages in the plant polysaccharide amylopectin, but has no action upon glycogen or glycogen limit dextrins.[81]



Manners [82] considers that the attack on the B chains will be similar, resulting once more in stubs of 2 or 3 D-glucose units. French has advanced the theory that the B chain stubs are only one or two D-glucose units long, which would bring the average length of the stubs down from 2·5 to 2 D-glucose units. This work was based upon the action of β-amylase on branched substrates, [83] whose structure after β-amylolysis could be easily determined.



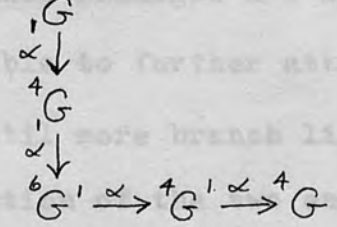
β-amylase-resistant arrangements.

non-resistant arrangements.

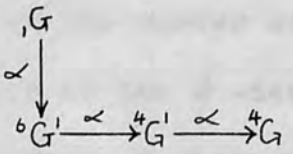
A = A chain,
B = B chain.

→ = α-(1→6)-glucosidic linkage.

The action pattern of α-amylase is essentially a random one, involving the hydrolysis of α-(1→4)-glucosidic linkages [84] to produce finally maltose and branched oligosaccharides of $\overline{DP} > 4$. With low concentrations of enzyme maltose, maltotriose and 6^3-α-maltosylmaltotriose are formed. [85]



With higher concentrations of enzyme a second stage of hydrolysis occurs; the maltotriose is hydrolysed to give maltose and glucose and the smallest dextrin produced is 6^3- α -glucosylmaltotriose:

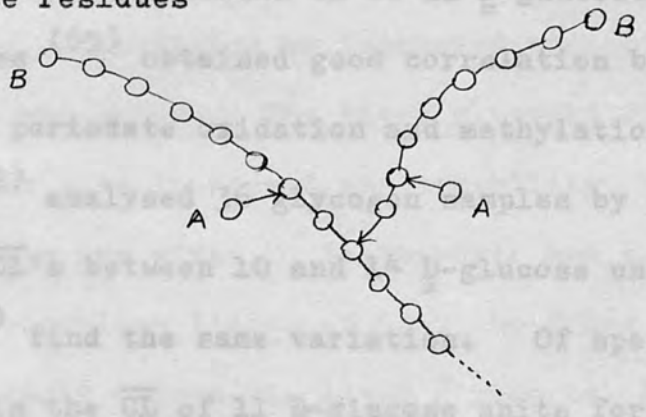


Panose has been observed in digests of Bacillus subtilis α -amylase with amylopectin.(84)

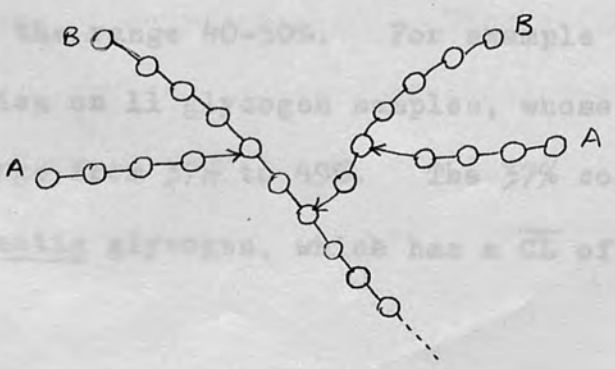
Manners and Wright(86) have recently developed a method for determination of the $\overline{CL}$ of glycogens, based on second stage α -amylolysis. Measurement of the apparent conversion to maltose (P_M) can be statistically related to the number of branch points and hence to the number of end groups. This method has now been modified (87) to enable 1mg. quantities of glycogen to be assayed.

Muscle phosphorylase in the presence of inorganic phosphate will hydrolyse 20-44% of the exterior chains of glycogen,(88,89) yielding D-glucose-1-phosphate and a limit dextrin (ϕ -dextrin). On addition of amylo-1,6-glucosidase (starch 6-glucanohydrolase) the outermost branch linkages are hydrolysed, and the molecule rendered susceptible to further attack by the phosphorylase, which proceeds until more branch linkages are encountered. Thus the concurrent action of the two enzymes produces complete

hydrolysis of the glycogen. The products are D-glucose-1-phosphate from phosphorylase action and D-glucose from the action of amylo-1,6-glucosidase.[88,90] The relative proportions of the products gives a measure of the number of branch linkages and hence the $\overline{CL}$.[91] The structure of the ϕ -dextrin was originally thought to contain A chain stubs of one D-glucose residue and B chain stubs of 6 D-glucose residues[90]



Whelan[92,93] and Illingworth[94,95] and their coworkers have modified the structure after finding that $\mathcal{R}$ -enzyme liberated maltotetraose on incubation with amylopectin ϕ -dextrin.[92] Also Whelan[92] could find no evidence for single D-glucose unit A chain stubs since no 6^3- α -glucosylmaltotriose was obtained on α -amylolysis. They suggested that the A and B chain stubs are 4 D-glucose units long, giving a structure as shown below:



The values of the average chain length ($\overline{CL}$) and average exterior chain length ($\overline{ECL}$) reported by various workers show that most glycogens have a $\overline{CL}$ of between 10 and 14 D-glucose units and an $\overline{ECL}$ of 7 to 9 D-glucose units. Results of methylation analyses by Haworth and ~~coworkers~~ Percival (43) Bell (44-48) and Hirst, Jones and coworkers (65) showed this variation in $\overline{CL}$, although most of the glycogens studied possessed a $\overline{CL}$ of 12 D-glucose units. Halsall, Hirst and Jones (65) obtained good correlation between $\overline{CL}$'s determined by periodate oxidation and methylation. Smith and Abdel-Akher (22) analysed 36 glycogen samples by periodate oxidation and reported $\overline{CL}$'s between 10 and 14 D-glucose units. Manners and Archibald (68) find the same variation. Of special note in this latter paper is the $\overline{CL}$ of 11 D-glucose units for a fetal pig liver glycogen ($[\alpha]_D$+191°). Bell and Manners (69) reported a $\overline{CL}$ of 13 D-glucose units for a fetal sheep liver glycogen, and Illingworth and coworkers (88) found a $\overline{CL}$ of 11·5 D-glucose units for a fetal guinea-pig liver glycogen. Unfortunately the fetal ages of the animals were not recorded, but it can be seen that the $\overline{CL}$ values are within the range encountered in all other mammalian glycogens.

The percentage of maltose liberated by the action of β-amylase is usually in the range 40-50%. For example Bell and Manners (69) reported studies on 11 glycogen samples, whose percentage conversions to maltose range from 37% to 49%. The 37% conversion was obtained from Helix pomatia glycogen, which has a $\overline{CL}$ of 7 glucose units —

well outside the normal range. The lowest % conversion for a mammalian glycogen was 41% for a human muscle glycogen.

Warren and Whittaker [96] have studied fetal goat liver glycogen through the period of gestation 95-146 days. They observed no correlation between $\overline{CL}$ or $\overline{ECL}$ and the age of the fetus. Most structures appeared to be normal, $\overline{CL}$ ranging from 12·1 to 15·7 D-glucose units and $\overline{ECL}$ from 8 to 12 D-glucose units. After 130 days of gestation an increased susceptibility to phosphorylase was noted. The liver glycogen from a mature goat did not exhibit this increased susceptibility, but no data on new-born animals was given. Illingworth and coworkers [88] reported phosphorylase limits of 42·7, 40·8 and 39·9% for fetal, new-born and adult guinea-pig liver glycogens, but no fetal age was quoted, and the results were not corroborated by tests on any more guinea-pigs. Several workers [97-99] have reported that glycogen in fetal guinea-pigs accumulates rapidly near term and decreases rapidly in the neonatal period, but Warren and Whittaker [96] give no figures for this in their study on goats.

Another group of enzymes which degrade glycogen (to D-glucose and a limit dextrin) are the mould amyloglucosidases (α-1,4-glucan glucohydrolases). They occur together with α-amylase, β-amylase, maltase (maltose 4-glucohydrolase) and transglucosylases in such moulds as Aspergillus niger [100,101] (it is known as glucamylase when obtained from this mould),

Rhizopus delemar,[102] Aspergillus oryzae,[103] and Clostridium acetobutylicum.[104] Barker and Carrington[105] reported the presence of an enzyme in A. niger "152", which produced D-glucose on incubation with starch. Barker and Fleetwood[100,106] developed the study of this enzyme using A. niger NRRL-330-1, and obtained an α-amylase-free enzyme preparation which attacked only substrates containing an α-D-(1$\rightarrow$4)-glucosidic linkage at the non-reducing end of the molecule, e.g. maltose, starch and amylopectin. The action on amylopectin ceased at 60% conversion, when branch points were encountered, although on prolonged incubation the traces of α-amylase rendered the molecule susceptible to further hydrolysis. A similar action on glycogen was noted. This work confirmed the observations of Kerr and coworkers,[101] who used the same strain of A. niger.

Pazur and Ando[107,108] have purified a commercial amyloglucosidase ("Diazyme") and studied its action on starch, amylopectin, amylodextrins and D-glucosyl oligosaccharides containing α-(1$\rightarrow$4)- and α-(1$\rightarrow$6)-linkages. They found that the purified enzyme hydrolyses α-(1$\rightarrow$4)- and α-(1$\rightarrow$6)-linkages, the former being hydrolysed more quickly. This results in almost complete hydrolysis of starch and amylopectin. The enzyme source was again A. niger, but the strain was not specified.

The amyloglucosidase from Rhizopus delemar has been studied by Phillips and Caldwell[102] and Tsiyisaka and Fukumoto,[109]

who respectively report 92% and 100% conversions of glycogen to D-glucose. Both this enzyme [110] and the corresponding one from A.oryzae [111] have been obtained in crystalline form. Hopkins and Kulka [112] have found an amyloglucosidase in Saccharomyces diastaticus which can bypass the α-(1 $\rightarrow$ 6)-linkages of amylopectin β-dextrin to produce a trisaccharide.

The function of glycogen as the body's reserve carbohydrate has long been known. The method of degradation of glycogen to blood sugar (D-glucose) was for a long time thought to be by hydrolysis, catalysed by an amylase-type enzyme. Glock in 1936 [113] reported an amylase in liver, which did not have a random action and produced maltose, which was further hydrolysed to glucose in most animal tissues. Parnas [114-116] at about the same time showed that glycogen degradation required inorganic phosphate, and Cori and Cori [117,118] identified the hydrolysis product as D-glucose-1-phosphate. Phosphorylase, (α-1,4-glucan: orthophosphate glucosyltransferase) the enzyme which catalyses the degradation of glycogen, has now been obtained pure from several muscle and liver sources.[119-124] It catalyses the reversible reaction,

$$\text{D-Glucose-1-phosphate} + [G]_n \rightleftharpoons [G]_{n+1} + H_3PO_4$$

(polysaccharide primer)

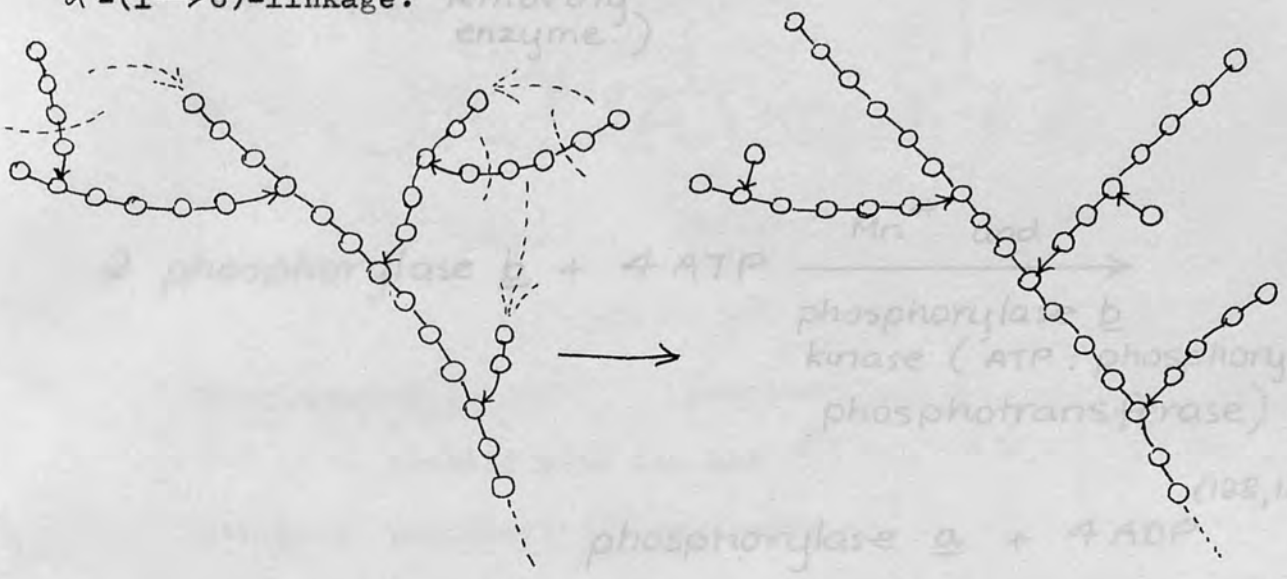
which led workers to believe that phosphorylase was responsible for the synthesis and degradation of glycogen. The synthetic

action requires a primer molecule, glycogen or amylopectin being the most efficient; amylose is less so. The corresponding plant enzyme, P-enzyme, has a less stringent primer requirement, needing only 4 (1 → 4)-linked α-D-glucose residues.[33,125] Maltotriose is a very poor primer, but maltotetraose, maltopentaose, maltohexaose, amylose and amylopectin make increasingly efficient primers.

The action of pure phosphorylase yields straight chains of α-(1 → 4)-linked D-glucose units, whereas liver homogenates synthesize both α-(1 → 4)- and α-(1 → 6)-linkages. This is due to the presence of a branching enzyme in liver. The resulting structure is glycogen-like in the latter case; in the former it approximates to an amylose-like structure. Similarly the degradation of glycogen by pure phosphorylase results in a 20-44% conversion[88,89] to D-glucose-1-phosphate and a φ-dextrin, whose structure is shown on p. 23. Liver preparations contain a debranching enzyme, amylo-1,6-glucosidase, which catalyses the hydrolysis of the α-(1 → 6)-linkage between a single D-glucose unit and a B chain in the φ-dextrin, and renders the molecule susceptible to further phosphorolysis.[88,89,90,126]

~~Brown,~~ Illingworth, Brown and Cori[94] have reported the presence of an oligo-1,4 → 1,4-glucantransferase activity in rabbit muscle closely associated with the amylo-1,6-glucosidase. Maltosyl or maltotriosyl units are transferred by this former enzyme to other

branches of the ϕ-dextrin molecule. This results in further branches with a single D-glucose residue attached by an α-(1→6)-linkage:



Amylo-1,6-glucosidase will cleave this new branch linkage. ~~Abdullah and~~ Abdullah and Whelan[93] and ~~Brown and Illingworth~~ Illingworth, Brown and Cori[95] have since jointly reported that this system will debranch glycogen itself, as well as the ϕ-dextrin obtained from it by phosphorylase action. As can be seen from the diagram, the oligo-1,4→1,4-glucantransferase is capable of producing a structure resembling the ϕ-dextrin structure put forward by Cori and Larner.[90] The debranching of glycogen by the transferase and amylo-1,6-glucosidase system can thus be readily understood.

Muscle phosphorylase occurs in two forms — an active form a and an inactive form b, which requires the addition of adenosine monophosphate (AMP) for activation. They may be interconverted as follows:

(127)

$$\text{phosphorylase } \underline{a} \xrightarrow[\text{(phosphate-removing enzyme.)}]{\text{PR enzyme}} 2 \text{ phosphorylase } \underline{b} + 4P_i$$

$$2 \text{ phosphorylase } \underline{b} + 4\,ATP \xrightarrow[\text{phosphorylase } \underline{b} \text{ kinase (ATP : phosphorylase phosphotransferase)}]{Mn^{++} \text{ and}}$$

(128,129)

$$\text{phosphorylase } \underline{a} + 4\,ADP$$

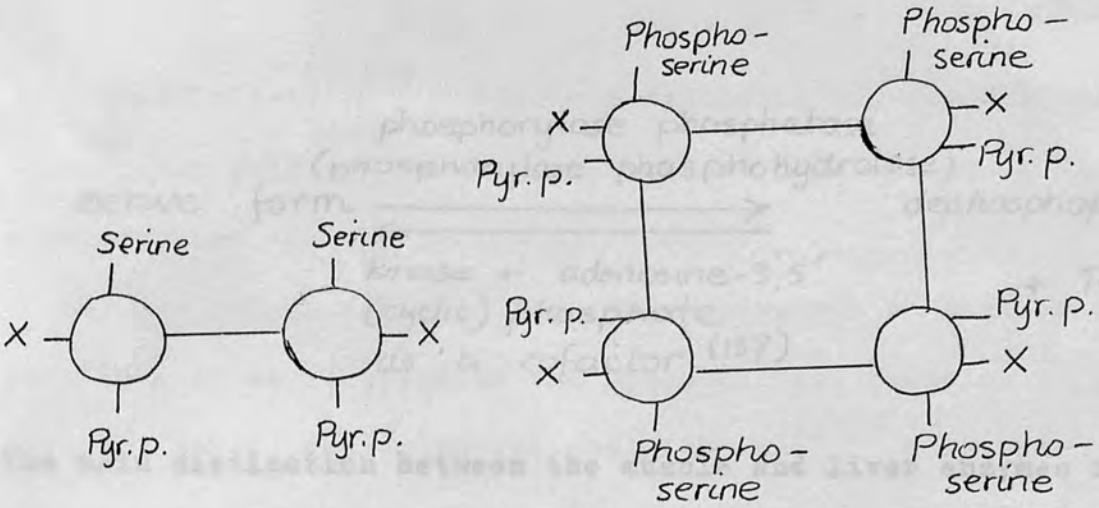
ATP = adenosine triphosphate

ADP = adenosine diphosphate

Muscle phosphorylase molecules are highly complex. The b form is a dimer containing in its protein chains the following amino acid sequence:

Lys - ~~Glutamine~~ $^{Glu(NH_2)}$ - Ileu - Ser - Val - Arg (130)

Activation of the enzyme involves addition of inorganic phosphate to the serine group to form phosphoserine, and the formation of a tetramer from two molecules of phosphorylase b. (128) The b form contains two binding sites for AMP, (121) whereas the a form has four.



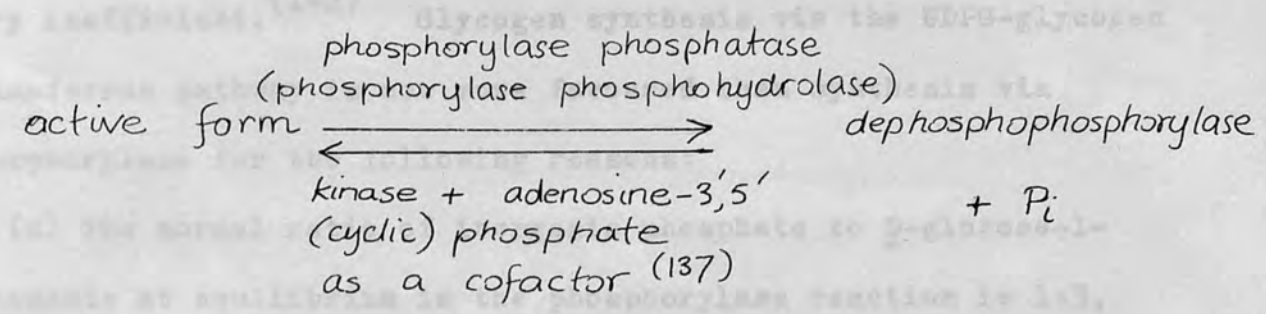
Phosphorylase b Phosphorylase a

X = binding site for AMP

pyr.p. = pyridoxyl 5-phosphate.

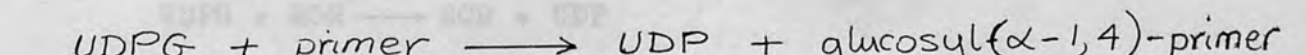
Both forms have been shown to contain pyridoxyl 5-phosphate, 4 moles per molecule of the a form, and 2 moles per molecule of the b form.[131,132] The function of the pyridoxyl 5-phosphate appears to be that of a prosthetic group, i.e. its presence is necessary for the activity of the enzyme. Some doubt has been expressed as to whether it is a true prosthetic group, since its reduction by sodium borohydride does not affect the activity of the enzyme.[133]

The liver phosphorylase is a very similar enzyme. It exists in an active form and an inactive one (dephospho-phosphorylase).[134] The latter form is not activated by AMP,[135] like phosphorylase b, but in the presence of Mg^{++} , ATP and a liver enzyme, dephosphophosphorylase kinase, (ATP: dephosphophosphorylase phosphotransferase) its activity is restored.[136]

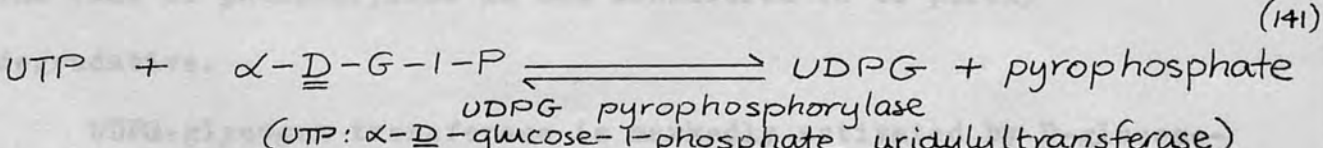


The main distinction between the muscle and liver enzymes is that there is no molecular weight change during conversion of the liver enzyme.(135)

In 1957 Leloir and Cardini (138) reported the synthesis of glycogen from uridine diphosphate glucose (UDPG) and a rat liver homogenate. Subsequently the enzyme concerned was found in rat muscle,(139) pigeon-breast muscle,(140) and rabbit brain.(9) The reaction is catalysed by an enzyme, UDPG-glycogen transferase (UDPglucose: α-1,4-glucan α-4-glucosyltransferase), which catalyses the transfer of an α-D-glucosyl unit from UDPG to an acceptor molecule such as glycogen:



The donor molecule arises from the reaction



Starch, ϕ- and β-dextrins make poor acceptor molecules for the rat liver enzyme.(138) Malto-oligosaccharides of $\overline{DP}$ 3-7 have been tested as acceptors for the rat muscle enzyme and are also

very inefficient.[142] Glycogen synthesis via the UDPG-glycogen transferase pathway is now more favoured than synthesis via phosphorylase for the following reasons:

(a) The normal ratio of inorganic phosphate to D-glucose-1-phosphate at equilibrium in the phosphorylase reaction is 1:3, whereas Larner, Villar-Palasi and Richman[143] have shown that glycogen synthesis occurs readily in normal and insulin-treated rat diaphragm muscle in conditions where this ratio was 300:1.

(b) The hormones glucagon and epinephrine stimulate glycogen breakdown only, although they produce this stimulation by increasing phosphorylase activity.[144]

(c) Glycogen synthesis still takes place in certain glycogen storage diseases, where phosphorylase is absent.[145,146]

(d) The biosyntheses of cellulose,[147] xylan[148] and starch[149] are known to take place through nucleotide intermediates, the general reaction being:-

$$UDPG + HOR \longrightarrow GOR + UDP$$

(G = glycosyl residue)

The role of phosphorylase is now considered to be purely degradative.

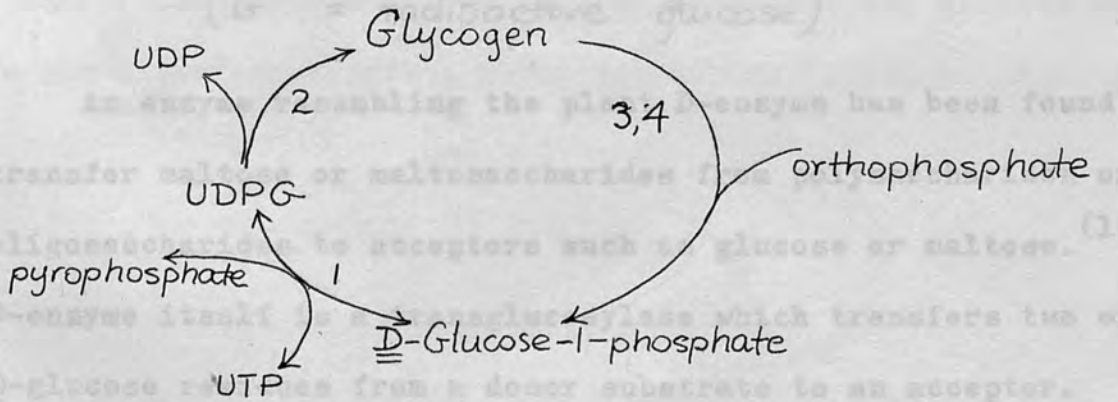
UDPG-glycogen transferase is markedly activated by D-glucose-6-phosphate[139,150] and insulin.[151-153] Two forms of the enzyme have been isolated.[154] One form (D) was found to be dependent on D-glucose-6-phosphate for its activity and the other

form (I) independent. The two forms are interconvertible

$$D \underset{\text{ATP and } Mg^{++}\text{ dependent phosphorylation}}{\overset{\text{dephosphorylation}}{\rightleftharpoons}} I \qquad (155, 156)$$

The branching enzyme amylo-1,4 → 1,6-transglucosidase has been studied by Larner,[157] who, using radioactive techniques, has shown that branching occurs when the exterior glycogen chains were between 6 and 11 D-glucose units long. Petrova has demonstrated an amylose isomerase in muscle.[158,159] It is distinctly different from the normal branching enzyme, since it can synthesize or degrade α-(1→6)-linkages in glycogen or starch, although it produces glycogens of normal structure.[160]

The synthesis and degradation of glycogen from D-glucose-1-phosphate may now be regarded as:



1. UDPG pyrophosphorylase.
2. UDPG - glycogen transferase + branching enzyme.
3. Phosphorylase.
4. Amylo-1,6- glucosidase + oligo-1,4 → 1,4 - glucantransferase.

In addition to this main scheme an ancillary pathway of degradation has been suggested in recent years by several workers.

A maltase (maltose 4-glucohydrolase) which also shows maltotransglucosylase (maltose 4-glucosyltransferase) activity has been reported by Giri and coworkers.[161] A series of α-(1 → 4)-linked oligosaccharides is produced from maltose by this enzyme. A similar activity has been observed in rat diaphragm.[162] Stetten[163] has proposed the following mechanism:

$$\text{Glucosyl-}G^{*} + \text{enzyme} \rightleftharpoons \text{Glucosyl-enzyme} + G^{*}$$

$$\text{Glucosyl-enzyme} + \text{glucosyl-}G^{*} \rightleftharpoons \text{Glucosyl-glucosyl-}G^{*} + \text{enzyme}$$

(G^{*} = radioactive glucose)

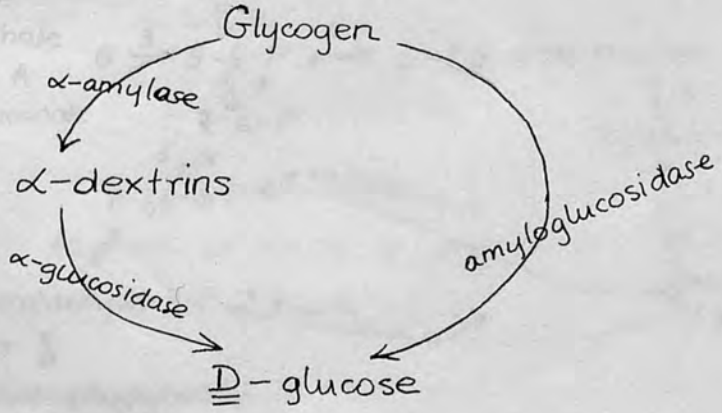
An enzyme resembling the plant D-enzyme has been found to transfer maltose or maltosaccharides from polysaccharides or oligosaccharides to acceptors such as glucose or maltose.[164-166] D-enzyme itself is a transglucosylase which transfers two or more D-glucose residues from a donor substrate to an acceptor. The donor can be maltotriose or higher maltosaccharides and the acceptor D-glucose, maltose or maltosaccharides:[167]

$$G\overset{1}{\rightarrow}\overset{4}{G}\overset{1}{\rightarrow}\overset{4}{G} + G\overset{1}{\rightarrow}\overset{4}{G}\overset{1}{\rightarrow}\overset{4}{G} \overset{\text{D-enzyme}}{\rightleftharpoons} G + G\rightarrow G\rightarrow G\rightarrow G\rightarrow G$$

maltotriose maltopentaose

Acid maltase has been found in human and rat livers.[168,169] No transferase activity was noted in the case of human liver. The enzyme is also capable of producing D-glucose from glycogen.[168-170] It appears to correspond to the enzyme reported in liver by Rosenfeld.[171,172] In its action it resembles the mould amyloglucosidases,[100,101] and has been assigned to this group by Torres and Olavarria.[170]

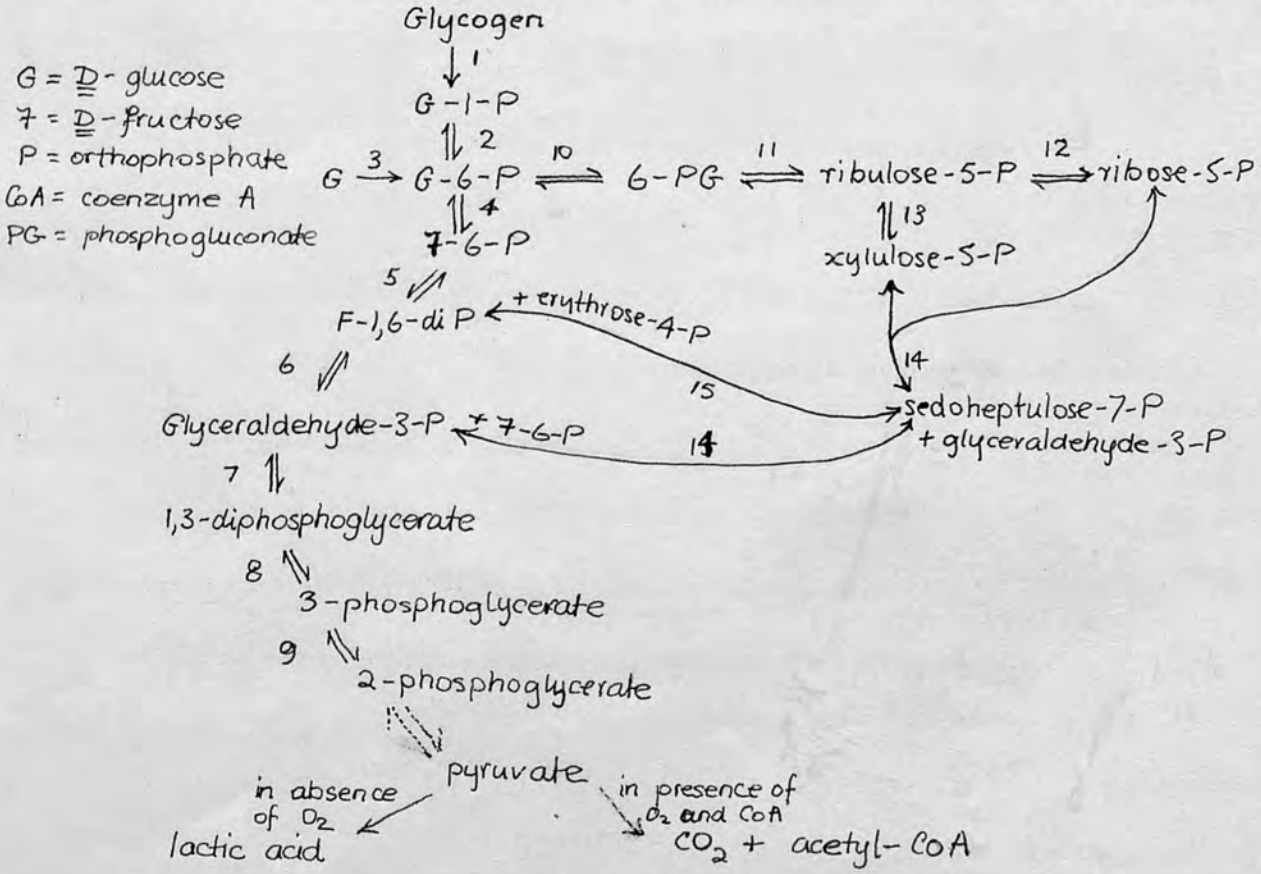
Another amylase found in liver possesses an α -amylase-type action. This enzyme has been studied by Torres and Olavarria,[173] Rutter and Brosemer[174,175] and Glock.[113,176] Malto-oligosaccharides are produced from glycogen by the α-amylase action. The action of the maltase, (or amyloglucosidase), produces D-glucose from these substrates. Rutter and Brosemer[175] do not consider that a combination of the α-amylase and maltase activities in the liver constitute a major pathway for glycogen degradation.



Stetten and Stetten[177] have suggested that the α-amylase could produce smaller glycogen molecules, which would then act as "seeds" or primers for further glycogen synthesis. They also

discuss the possibility that the transglucosylation reactions, which result in no net synthesis or degradation of glycosidic bonds, serve to redistribute the bonds, thus causing the variation of molecular size and branching characteristics so consistently observed in glycogens. Stetten and Stetten [178,179] have demonstrated the constant turnover of glycogen in the liver by means of injection of radioactive glucose into the body.

The conversion of hexose monophosphates to a form of energy can follow two main pathways — glycolysis or the pentose phosphate pathway.[180] It has been demonstrated that carbohydrate degradation in mammals occurs by both pathways.[181] Glycogen can also be synthesized by reversal of these two pathways, intermediary metabolites such as D-ribose, D-xylose,[182] pyruvate, lactate and carbon dioxide [183] being incorporated into glycogen.



1. phosphorylase + amylo-1,6-glucosidase
2. phosphoglucomutase (D-glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase)
3. hexokinase (ATP: D-hexose 6-phosphotransferase)
4. phosphoglucoisomerase (D-glucose-6-phosphate ketol-isomerase)
5. phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase)
6. aldolase (ketose-1-phosphate aldehyde-lyase)
7. phosphoglyceraldehyde dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase [phosphorylating])
8. phosphoglycerate kinase (ATP: D-3-phosphoglycerate 1-phosphotransferase)
9. phosphoglyceromutase (D-2,3-diphosphoglycerate: D-2-phosphoglycerate phosphotransferase.)
10. glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase)
11. 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate: NAD oxidoreductase)
12. phosphoribose isomerase (D-ribose-5-phosphate ketol-isomerase)
13. phosphoribulose epimerase (D-ribulose-5-phosphate 3-epimerase)
14. transketolase (D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate dihydroxyacetonetransferase)
15. transaldolase (D-sedoheptulose-7-phosphate,: D-glyceraldehyde-3-phosphate glycolaldehydetransferase)

The four enzymes in these systems most closely related to glycogen metabolism are glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase), phosphoglucomutase (D-glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase), phosphoglucoisomerase (D-glucose-6-phosphate ketol-isomerase) and hexokinase (ATP: D-hexose 6-phosphotransferase).

Hexokinases occur in all cells where metabolism involves free sugars. They catalyse the transfer of one mole of phosphate from ATP to the sugar, forming a sugar phosphate. They have been demonstrated in mammalian brain,[184] liver,[185] and muscle.[186] Rat liver contains a specific glucokinase and fructokinase.[185] Recently Viñela [187] has demonstrated a specific glucokinase and a non-specific hexokinase in rat liver. The glucokinase will phosphorylate D-glucose and D-mannose, forming D-glucose- and D-mannose-6-phosphates respectively.

Phosphoglucomutase, which catalyses the interconversion of D-glucose-1-phosphate and D-glucose-6-phosphate, was first noted by Colswick and Cori.[188,189] It is activated by D-fructose-1, 6-diphosphate.[190] Leloir and coworkers showed that phosphoglucomutase required a coenzyme which was present in impure D-fructose-1,6-diphosphate [191] and D-glucose-1-phosphate. The coenzyme was found to be D-glucose-1,6-diphosphate.

$$G\text{-}1\text{-}P + \underset{(\text{phospho-enzyme})}{PGM} \rightleftharpoons G\text{-}1,6\text{-}diP + \text{dephospho-enzyme}$$

$$G\text{-}1,6\text{-}diP + \text{dephospho-enzyme} \rightleftharpoons G\text{-}6\text{-}P + \underset{(\text{phospho-enzyme})}{PGM}$$

The phosphorus occurs as phosphoserine, in the ratio of one atom of phosphorus per molecule of enzyme.[192]

Phosphoglucoisomerase [193] catalyses the conversion of D-glucose-6-phosphate to D-fructose-6-phosphate.

Phosphomannoisomerase [194] is a very similar enzyme, giving the same end product from α-D-mannose-6-phosphate. Both mechanisms proceed through cis- or trans-ene-diol intermediates.[195]

Glucose-6-phosphatase is one of the many phosphatases occurring in the liver. It has a much higher specificity than the acid or alkaline phosphatases.[196] It will catalyse the hydrolysis of α-D-glucosamine-6-phosphate [197] but has no action on D-glucose-1-phosphate, D-fructose-6-phosphate or β-glycerophosphate.[196]

A lack of one or more of the enzymes concerned with glycogen metabolism leads to the manifestation of glycogen storage diseases. The diseases are characterised by abnormally large accumulations of normal glycogen, or normal accumulation of glycogens with abnormal structures, in various body tissues. A classification of the diseases has been made possible, since most of the particular enzymic lesions are now known.[198,199] Cases have been reported where more than one member of a family has suffered from the same type of the disease,[168,200,201] which implies that a single recessive gene is responsible. Each type of the disease is considered to be caused by a distinct recessive gene, although Manners [11,202] has studied a case of siblings, one of whom suffered from Type I of the disease, whilst the other suffered from Type III.

Type I or von Gierke's disease is the most common type [203]

and also the first to be reported.[204] The characteristics of the disease are abnormally high glycogen concentration in the liver and sometimes the kidney. Normally administration of glucagon, epinephrine, fructose or galactose causes a rise in the blood sugar level, but no such increase is observed in Type I patients.[205,206] The glycogen has a normal structure,[200] but the glucose-6-phosphatase activity is practically negligible.[11,207] This causes the observed accumulation of glycogen, and the lack of rise in blood sugar, and has no effect upon the glycogen structure.

Type II or Pompe's disease is the most lethal form, patients normally dying before 18 months of age.[198] Glycogen is deposited in all tissues, especially heart, muscle and liver. The structure appears to be normal.[208] No plausible or consistent explanation was put forward until Hers measured the acid α-(1$\rightarrow$4)-glucosidase (maltose 4-glucohydrolase) activity in the liver, and found it to be strikingly low.[168] This α-(1$\rightarrow$4)-glucosidase was found to be associated with the lysosomes,[169] which contain powerful hydrolases capable of destroying phosphorylase. Thus, in the lysosomal regions, the pathway involving α-(1$\rightarrow$4)-glucosidase and α-amylase may be the only one available for glycogen degradation, and a low α-(1$\rightarrow$4)-glucosidase activity would then result in glycogen accumulation. The clinical manifestations of the disease are in

agreement with this interpretation. The deposition of the glycogen in distinct granules, which has been noted, suggests a very localised enzymic lesion, and the fact that the general carbohydrate metabolism seems undisturbed [199] could arise from normal metabolism in the rest of the cytoplasm.

Type III or Cori's disease is characterised by deposition of glycogen in liver, muscle and heart tissues. The glycogen has a limit-dextrin structure.[200] Manners [202] and Polglase and coworkers [208] have studied similar cases. The abnormality was shown to be due to a deficiency in amylo-1,6-glucosidase,[201] the phosphorylase activity being normal.[209] Phosphorylase can thus produce a ϕ-dextrin, which cannot be further degraded, due to the debranching enzyme deficiency. There is a very low rise in blood sugar after adrenalin injection, but it is possible to keep patients alive by controlling the diet, especially the carbohydrate intake.[199]

Type IV or amylopectinosis has been found in only one patient to date.[210] Cori [198,200] examined the liver glycogen, and found an extremely low degree of branching and a crystalline form. The glycogen-iodine complex was a violet colour. These facts indicated that the polysaccharide had an amylopectin-like structure. It was presumed that a branching enzyme deficiency caused the abnormality although no experimental evidence was given. Cirrhosis of the liver occurred in this case, due to the deposition of foreign material.[199]

Only two cases of Type V disease have been reported.[203,211] A deposition of glycogen of normal structure occurs in the muscle fibres. No definite enzymic lesion has been assigned to this disease as yet. In the second case [211] there is a marked decrease in phosphoglucomutase activity (5% of normal) and glycolysis was only 25% of normal.

Type VI(a) disease [209] is caused by liver phosphorylase deficiency; the liver glycogen structure is normal, but large amounts accumulate. The muscle glycogen and phosphorylase are normal. This case provides evidence for the fact that liver and muscle phosphorylases are two distinct enzymes, and also indicates that phosphorylase is not of major importance in glycogen synthesis.

The Type VI(b) disease is caused by muscle phosphorylase deficiency and results in abnormally large deposition of glycogen in muscle tissues. Cases have been reported by Mommaerts,[212] Schmid [145] and McArdle.[213] Phosphoglucomutase (D-glucose-1, 6-diphosphate: D-glucose-1-phosphate phosphotransferase), UDPG-glycogen transferase (UDPG: α-1,4-glucan α-4-glucosyltransferase), and UDPG-pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase) were all normal,[146] except that in the second case [145] the UDPG-glycogen transferase activity was only half the normal value. Other reports of low transferase activities [214] in Type I and Type III diseases may point the way to an alternative synthetic pathway not involving the hexokinase and phosphoglucomutase reactions.[215]

The carbohydrate metabolism of the human fetal tissues has not been studied so extensively as that of the avian and amphibian embryos. Rat and guinea-pig fetuses are most commonly used in the mammalian classes.

In the adult liver there is a marked response to hormonal stimuli. Glucagon and epinephrine stimulate glycogenolysis, thus causing a rise in the blood sugar level (hyperglycaemia). The hormones stimulate the production of adenosine-3′ 5′ (cyclic) phosphate, which in turn promotes the conversion of inactive dephosphophosphorylase to active phosphorylase.[216] In addition epinephrine has been found to repress UDPG-glycogen transferase activity,[217] thus effectively blocking the pathway for glycogen synthesis. Insulin causes hypoglycaemia by stimulating glycogenesis, thereby removing glucose from the blood. The glycogenesis appears to take place in muscle,[218] although de Duve and his coworkers have supported the view that insulin causes primarily liver glycogenesis.[219-221] The adrenal hormone, adrenocorticotropic hormone (ACTH), exerts an influence on adrenal phosphorylase analogous to that of glucagon and epinephrine on hepatic phosphorylase.[129]

The stimulus of glycogen synthesis in the fetus by hormones has been studied fairly extensively, notably by Jost, Jacquot and coworkers.[222,223] A sudden onset of glycogen storage in the fetal liver was noted on the 19th day in the case of the rat,[222]

and on the 25th day in the case of the rabbit. By means of hypophysectomies on the fetuses and adrenalectomies on the fetuses and mothers, the sources of hormonal stimuli were removed. Glycogen synthesis was found to be stimulated by the adrenal corticosteroids, which are controlled by the hypophysis. Maternal adrenal secretions are initially responsible for glycogen synthesis in the fetus, the rôle being gradually assumed by the fetal counterparts.(223) After the sudden onset of glycogen synthesis the hypophysis has very little effect, probably because the adrenals have by then received a large enough stimulus to proceed without any further control.(222)

Chick embryos store glycogen at a very early stage; for example Novikoff and coworkers (224) have demonstrated its presence after 3 days, together with such substrates as glucose-6-phosphate, hexose-diphosphate, phospho-glyceric acid, etc., indicating the operation of the phosphorylating glycolysis pathway. Glycolysis has been observed in human fetuses aged 8 to 25 weeks.(225) The tissues are well differentiated biochemically at the beginning of this period. The existence of the pentose phosphate pathway in tissues of the same fetal ages have also been shown.(226)

Several workers (7,8,227,228) have observed that the placenta initially assumes the rôle of the fetal liver, transporting carbohydrate material from the maternal to the fetal tissues. As the fetus begins to accumulate hepatic glycogen this placental

function gradually ceases. Villee [225] quotes 12 weeks as the duration of the placental function in human fetuses.

Demonstration of glycogen in human fetal tissues at fairly early stages depends exclusively upon histochemical techniques. In general it has been found to occur more abundantly in tissues other than hepatic ones up to 7 or 8 weeks.[229-232] In most cases it appears to be concentrated in the epithelial layers of various parts of the embryo. Mackay and his coworkers [230-232] consider that it takes part in the promotion of rapid growth in these areas. Shapovalov has found traces of glycogen in the liver cells of 6, 7 and 8 week fetuses.[229] Mackay [232] and his coworkers have found more evidence in support of the fact that the yolk sac (placenta) functions in place of the liver in the early embryonic stages. Its epithelial cells contain glycogen and it is connected to the liver by a vascular system. In the horizons XIII and XIV [233] (23-30 days) they noted the beginnings of the shift of function from yolk sac to liver, when 5-nucleotidase and non-specific esterase activities became evident in liver cells for the first time. Kitamura [234] reports that glycogen is first apparent in the human fetal liver at 4 months, and that it rises to a maximum in the 6th to 9th months. It disappears abruptly at term, but with premature births there is no such decrease. In contrast Lelong and coworkers [235] could demonstrate no hepatic glycogen in human fetuses until just before term, although it was present in lung tissue throughout fetal life, until the onset of

hepatic glycogen storage.

The sudden accumulation of glycogen in the fetus previously mentioned [97,98] has been observed in most mammals just prior to term. Dumm,[236] Dawkins,[99] and Weber and Cantero [237] have studied rat fetuses; Nemeth, Insull and Flexner,[97] Hard, Reynolds and Winbury [238] and Ducommun-Lehmann [239] have studied guinea-pigs and Shelley [240] and Dawkins [99] sheep. In all cases these workers have noted that the rapid increase in glycogen content is followed by a sharp decrease a few hours after birth. The process of building up the glycogen reserves to the adult level may take several days. With two-day premature births the same pattern was found.[99]

Nemeth [241] has attributed this sudden decrease in glycogen content to the appearance of glucose-6-phosphatase activity a few days before birth. This activity increases greatly immediately after birth,[99] thus causing the depletion of glycogen.

The human fetus appears to possess low glucose-6-phosphatase activity at 11 weeks [225] on the other hand. Villee reports that by 22 weeks it has reached half the normal adult level.[227] The work of Auricchio and Rigillo [242] points to a low and fairly constant activity in 5-9 month fetuses. They could show no sudden increase in enzyme activity corresponding to the normal mammalian pattern.

The mammalian fetus has been shown to possess many of the

enzymes involved in glycogen synthesis. Nemeth [97] and Burch and coworkers [243] have found fairly high phosphorylase activity in the guinea-pig and rat fetuses in the last few days of gestation, while Kornfeld and Brown [244] observed an increase to 3 times the normal level of the adult guinea-pig in the period between the 65th day of gestation and the second postnatal day. By the 6th postnatal day it had fallen to the normal adult level.

Phosphoglucomutase activity is high in fetal guinea-pig [97] and rat [237] livers, as is phosphohexoisomerase in the fetal and newborn rat liver.[237,243]

Hexokinase activity increases in the fetal guinea-pig liver,[245] until on the 50th day it is as high as the normal adult level. During this time the placental activity decreases correspondingly. The activity in the fetal rat liver decreases markedly from the 5th day prior to term onwards, [243] accompanied by a decrease in phosphofructokinase activity. This is indicative of a fall in the high rate of glycolysis found in the fetus.[237,246] Coupled with the high phosphorylase activity and the appearance of glucose-6-phosphatase prior to term, it appears to favour glycogen conversion to glucose rather than lactate.[243]

UDPG-glycogen transferase (UDPG: α-1,4-glucan α-4-glucosyltransferase) and UDPG pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase) activities have been measured in the fetal guinea-pig [244] and were found to increase from 45 days to term, when they reach the adult level.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP* oxidoreductase) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate: NAD^+ oxidoreductase) activities reach the adult level by term, but then decrease for 3 weeks.[243,237,247] Burch and coworkers have attributed this to the fact that the enzymes increase in activity in the presence of a high carbohydrate concentration. However, during the 3 week nursing period, the only food given is milk, which has a high fat rather than carbohydrate content.

*NADP = nicotinamide adenine dinucleotide phosphate.

$^+$NAD = nicotinamide adenine dinucleotide.

EXPERIMENTAL

Solvents used for chromatography

I. ethyl acetate: acetic acid: water [248] 9: 2: 2 by volume

II. ethyl acetate: pyridine: water [249] 10: 4: 3 " "

III. n-butanol: ethanol: water [250] 40:11:19 " "

IV. n-butanol: pyridine: water [251] 6: 4: 3 " "

V. acetone: water [106] 1: 1 " "

Sprays used for locating compounds

I. p-anisidine-hydrochloride, 2%(w/v) in n-butanol.[252]

II. 0.93g. aniline and 1.66g. phthalic acid in 100ml. n-butanol.[253]

III. silver nitrate solution in acetone, followed by ethanolic sodium hydroxide.[254]

IV. Ninhydrin, 0.4% in n-butanol (95%).[255]

Samples used

A. Urine samples taken from patient G.B. when fed on a diet containing amylopectin.

B. The above sample de-ionised with Biodeminrolit (carbonate form).

C. Sample of lactose-reduced milk.

D. Urine samples taken from G.B. when fed on a diet containing amylose and lactose-reduced milk.

Experiment 1.

Extraction of glycogen with alkali

Liver tissue was stored at -15° until required for extraction of glycogen. Liver tissue in the frozen state was ground into small pieces and added to a 30%(w/v) solution of potassium hydroxide in water (1g. of tissue to 9ml. of potassium hydroxide solution). The mixture was heated at 100 for 2-3 hr. or until well-digested. After removal of any solid material the solution was cooled and glycogen precipitated with ethanol. Nitrogenous material and phosphate were removed from the glycogen by the procedure of Somogyi.(256)

Experiment 2.

Extraction of glycogen with trichloracetic acid

Liver tissue was homogenised with ice-cold 10% (w/v) trichloracetic acid for 5 min. After centrifuging at 0° the supernatant liquid was kept, and the remaining tissue re-extracted four more times with trichloracetic acid. The glycogen was precipitated from the pooled supernatants by 5 volumes of ethanol. After standing overnight at 4° the glycogen was centrifuged, washed three times with ethanol and once with ether and dried under reduced pressure in the presence of phosphorus pentoxide.

Experiment 3

Determination of purity of glycogen

Glycogen (ca. 5mg.) was hydrolysed with 2N-sulphuric acid

(ca. 2ml.) for 2 hr. at 100°. After cooling, the solution was neutralised with N-sodium hydroxide and the volume made up to 25 ml. with distilled water. 4 ml. aliquots were assayed for glucose content by the method of Somogyi.(257) The purity of the glycogen was expressed as a percentage of glucose found in a known weight of glycogen. The results obtained on fetal and other glycogens are given in Tables I and VI.

Experiment 4.

Determination of β-amylase activity

The activity of sweet-potato β-amylase was measured using the method of Hobson, Whelan and Peat,(258) except that the temperature of incubation was 30°. The activity was defined as the number of mg. maltose liberated by 1 mg. enzyme under the above conditions.

Experiment 5.

Incubations of glycogen with β-amylase

Glycogen (30 mg.), β-amylase (1800 units), and 0·2M-sodium acetate buffer pH 4·6 (5ml.), in a total volume of 25ml., were incubated at 30° for 24 hr. The maltose liberated was determined by the method of Somogyi.(257) A portion of the digest was examined by descending chromatography on Whatman No.1. paper in solvent I, and the sugars located with spray I.

Experiment 6.

Glycogen (ca. 1mg.), β-amylase (50-70 units) and 0·2M-sodium acetate buffer pH 4·6 (0·4ml.), in a total volume of 10ml., were

incubated at 30° for 24 hr. 4ml. of the digest was removed and diluted to 50ml. with water. Aliquots of 2ml. were assayed for maltose content by the method of Parke and Johnson.(259) The absorptions were measured on a Hilger "Spekker" at 710 mμ using an Ilford No. 609 filter. The proportions of the constituents of individual digests and the amount of dilution required varied with the amount available of each glycogen sample.

Experiment 7.

Preparation of α-amylase

α-amylase was prepared from human saliva by the method of Fischer and Stein.(260) The crystallisation procedure was omitted and the enzyme freeze-dried in 0·2M-sodium citrate buffer pH 7·0.(261) The activity was measured by the method of Manners and Wright,(86) one unit of α-amylase activity being defined as the quantity of enzyme which liberates 1mg. of apparent maltose (measured in this case by the Somogyi reagent) from 1ml. of 1% starch in 3min. at 35°.

Experiment 8.

Incubation of maltose with α-amylase

α-amylase preparation (1mg.), maltose (5mg.), and 0·5% sodium chloride (1ml.), in a total volume of 10ml., were incubated at 35°. Samples were taken at hourly intervals from 1 to 12 hr., then at 24 and 30 hr. and examined by descending chromatography on Whatman No.1. paper in solvent I. Sugars were located with spray I.

Experiment 9.

Incubation of glycogens with α-amylase

Glycogens of known chain length were incubated with α-amylase to check that the particular preparation conformed with the equation derived by Manners (86) for the calculation of $\overline{CL}$. Digests contained glycogen (12·5 mg.), α-amylase (50 units) and 0·5% sodium chloride in a total volume of 25ml. and were incubated at 35° for 15 hr. The apparent percentage conversion to maltose was measured by the method of Somogyi.(257) A portion of the digest was evaporated under reduced pressure to a small volume and examined by descending chromatography in solvent I using Whatman No.1. paper. The sugars were located with spray I.

Experiment 10.

Glycogens of known and unknown $\overline{CL}$ (0·5 - 1·5 mg.), 0·5% sodium chloride (0·2ml.) and α-amylase (2-6 units), in a total volume of 10ml., were incubated at 35° for 15 hr. After appropriate dilution, the maltose content was measured by the method of Parke and Johnson.(259) Chromatographic investigation of the digests was as described in Experiment 9.

Experiment 11.

Periodate oxidation of glycogens

Glycogen was oxidised with potassium metaperiodate at room temperature by the method of Bell and Manners (69) and by sodium metaperiodate by the modification (68) of the method of Potter and

Hassid.[66] In most cases the quantity of glycogen used was in the region of 50mg. and the digests were reduced in scale accordingly.

Experiment 12.

Incubation of glycogen with glucamylase

Rabbit liver glycogen (29.7mg.), glucamylase solution* (5ml.) and M-sodium acetate buffer pH 4.05 (2ml.), in a total volume of 25ml., were incubated at 50°. Aliquots of 2ml. were withdrawn at time intervals of 2, 4, 10, 19½, 21 and 26 hr. and the glucose formation measured by the method of Somogyi.[257]

Experiment 13.

Combined reaction of β-amylase and glucamylase on glycogen

Rabbit liver glycogen (116.2mg.), 0.2M-sodium acetate buffer pH 4.6 (20ml.), and β-amylase (ca. 7000 units) in a total volume of 100ml. were incubated at 30°. Aliquots of 2ml. were removed after 24, 44 and 48 hr. intervals. The maltose formed was measured by the Somogyi reagent.[257] A portion (25ml.) of the digest was incubated at 50° with 5ml. glucamylase solution. Aliquots of 2ml. were withdrawn after 47, 116 and 119 hr. and the glucose formed measured by the Somogyi reagent.[257] A portion of this digest was evaporated under reduced pressure to a small volume and examined by descending chromatography on Whatman No.1. paper in solvent I. The sugars were located with spray I.

* preparation by Dr. J.G.Fleetwood

Experiment 14.

Isolation of limit-dextrins

Baboon liver glycogen (130·2mg.), 0·2M-sodium acetate buffer (20ml.) and β-amylase (7800 units), in a total volume of 100ml., were incubated at 30° for 48 hr. to allow for completion of enzyme action. Aliquots of 2ml. were removed and the maltose formed measured by the method of Somogyi.(257) The remaining solution was dialysed overnight against running tap water and reduced in volume by evaporation under reduced pressure. Protein was removed from the solution by shaking for 1 hr. with a mixture of 0·25 volumes of chloroform and 0·1 volumes of amyl alcohol.(262) The aqueous layer was dialysed again and evaporated to a small volume as before. The limit dextrin was precipitated with ethanol, washed 3 times with ethanol and once with ether and dried under reduced pressure in the presence of phosphorus pentoxide. The purity of the dextrin was determined by the procedure described in Experiment 3.

Experiment 15.

Baboon glycogen (121·5mg.), M-sodium acetate buffer pH 4·05 (8ml.) and freeze-dried glucamylase preparation* (60mg.), in a total volume of 100ml., were incubated at 50° for 48 hr. Aliquots of 3ml. were withdrawn and the glucose formed measured by the method of Somogyi.(257) The isolation of the limit dextrin followed the procedure described in Experiment 14.

* preparation by Dr. J.G.Fleetwood

Experiment 16.

Periodate oxidation of dextrins

Glycogen β-dextrin (30mg.) was oxidised with 8% (w/v) sodium metaperiodate (1ml.) in a total volume of 10ml. under the conditions of Manners and Archibald.(68)

Experiment 17.

Glycogen glucamylase limit-dextrin (29·2mg.) was treated as described in Experiment 16.

Experiment 18.

Incubation of dextrins with α-amylase

Glycogen β-dextrin (1·1mg.), α-amylase (4 units) and 0·5% sodium chloride (0·2ml.), in a total volume of 10ml., were incubated at 35° for 15 hr. After dilution aliquots of 2ml. were assayed for maltose by the method of Parke and Johnson.(259)

Experiment 19.

Glycogen glucamylase limit-dextrin (0·9mg.) was incorporated into a digest and the maltose liberated determined as in Experiment 18.

Experiment 20.

Incubation of rabbit liver glycogen with "Diazyme".

"Diazyme" (1mg.), glycogen (29·6mg.) and M-sodium acetate buffer pH 4.05 (2ml.), in a total volume of 25ml., were incubated at 50°. After 24 hr. the glucose production was measured by the Somogyi reagent.(257)

Experiment 21.

Separation of enzymic activities of "Diazyme"

A saturated solution of "Diazyme" in 1ml. of water was applied in a streak to Whatman No.3. paper. Descending chromatography in solvent V was carried out for 7 hr. After drying in cold air, the amylolytic activities were located by cutting strips 3cm. wide from each side of the paper and incubating 1cm. portions of these with 0·05% amylose solution (1ml.) and 0·02M-sodium phosphate (pH6·18)-0·03M-sodium chloride buffer (5ml.) for 6 hr. at 35 . Aliquots of 5ml. were added to N-sulphuric acid (1ml.) and 0·006% iodine solution (1ml.). The amylolytic bands were characterised by a marked decrease in the blue colour of the iodine-amylose complex.

Experiment 22.

The portions of the paper showing amylolytic activity were eluted with the phosphate-chloride buffer. The eluant (5ml.), glycogen (30mg.) and M-sodium acetate buffer pH 4·05 (2ml.), in a total volume of 25ml., were incubated at 50° for 24 hr. and the glucose production measured by the Somogyi reagent.(257)

Experiment 23.

Measurement of the absorption spectra of glycogen-iodine complexes

Glycogen (2·5mg) in 2·5 ml. water and 2·5 ml. iodine solution (0·2% iodine and 2% potassium iodide in water) were made up to 25 ml. with water, adding one drop of 6N-hydrochloric acid. The absorption

of the glycogen-iodine complex was recorded in the spectral region 400 - 700 mμ on the Unicam S.P.500, using 1cm. cells. The reference solution was a 0·02% iodine, 0·2% potassium iodide solution.

Experiment 24.

Extraction of concanavalin A

Concanavalin A was extracted from jack-bean meal with a 0·9% sodium chloride solution as described by Cifonelli and Smith.(263) No polyvinyl alcohol was added to the extract, but all subsequent determinations were carried out on the same day, under identical conditions.

Experiment 25.

Measurement of glycogen values

Glycogen values were measured according to the method described by Smith and coworkers.(41) The turbidity was measured at 430 mμ on a Hilger "Spekker", using an Ilford No. 601 filter. The standard glycogen used was a sample of calf liver glycogen. Some results are given in Table VII.

Experiment 26.

Partial acid hydrolysis

Trace precipitates of glycogen obtained from human fetal livers of ages 11½, 12, 13-13½ and 14½-15 weeks were subjected to partial acid hydrolysis with 0·05N-sulphuric acid for 8 hr. according to the conditions of Wolfrom, Lassetre and O'Neill.(58) The hydrolysates were examined by descending chromatography in solvent I

on Whatman No.1. paper and the sugars located with spray I. Samples of baboon liver glycogen and fetal liver glycogen (26 weeks) were treated in the same manner. The hydrolysates of the latter two were reduced with potassium borohydride and the borohydride removed by several treatments with methanol. The hydrolysates were then examined by electrophoresis on Whatman No.3. paper in 0·062M-sodium molybdate buffer (264) for 45 min. at 21 v/cm. The sugars were located with spray III.

Experiment 27.

Measurement of glucose-6-phosphatase activity

Glucose-6-phosphatase activity in liver was determined by the method of Calderbank, Kent and coworkers, (11) using dipotassium glucose-6-phosphate as substrate in 0·1M-potassium citrate buffer pH 6·5. The inorganic phosphate liberated after 10, 20 and 30 minutes' incubation at 35° was determined by the method of Fiske and Subbarow. (265) Results were expressed as μMoles of phosphate liberated per hr. per g. frozen tissue.

Experiment 28.

Measurement of phosphoglucomutase activity

Phosphoglucomutase activity was measured by the method of Najjar (266) using 0·02M-dipotassium glucose-1-phosphate as a substrate and 0·05 M-cysteine pH 7·5 with 6mM-magnesium sulphate as the buffer. Incubations were carried out at 35° for 5, 10 and 15 min. The inorganic phosphate was liberated by hydrolysis with

sulphuric acid and measured as in Experiment 27. Activities were expressed as μMoles of hydrolysable phosphate used per hr. per g. frozen tissue (see Table IV).

Experiment 29.

Measurement of phosphoglucoisomerase activity

Phosphoglucoisomerase activity was determined by a slight modification of the method of Glock and ~~McLean.~~ coworkers (267) The reaction mixture contained 1 μmole dipotassium glucose-6-phosphate in 0·4ml. 0·06M-Tris pH 9·0 and 0·1ml. liver homogenate. The fructose-6-phosphate formed was measured colorimetrically by the ethanolic resorcinol reagent of Roe.(268) Results were expressed in units of activity per mg. frozen tissue, one unit being defined as the quantity of enzyme that produces 1 μMole of fructose-6-phosphate per min. at 37°. (See Table V).

Experiment 30.

Examination of sugars in sample A

The samples were examined by descending chromatography in solvents I, II, III and IV on Whatman No.1. paper. Sugars were located with sprays I, II and IV.

Experiment 31.

Samples A and B were examined by electrophoresis on Whatman No.3. paper in 0·1M-sodium borate (269) buffer pH 10·0, at 30 V/cm. for 1 hr. Sugars were located with spray III.

Experiment 32.

The reducing sugars in sample B were reduced to the corresponding alcohols with potassium borohydride. They were examined by electrophoresis on Whatman No.3. paper in 0·062M-sodium molybdate buffer pH 5·0 at 21 v/cm. for 45 min. Sugars were located with spray III.

Experiment 33.

Sample B was heated at 100° for 3 hr. with Amberlite IR-120 (H+ form) ion exchange resin in order to hydrolyse the sugars. The resin was filtered off, washed with water, and the combined washings and filtrate evaporated under reduced pressure until almost dry. The solution was examined by descending chromatography on Whatman No.1. paper in solvent II and the sugars located with spray I.

Experiment 34.

A portion of sample A was placed in a streak on Whatman No.3. paper and chromatographed in solvent II. The position of the isomaltose band was located by spraying strips cut from each side of the paper with spray I. The band was cut out and the sugar eluted with water. The resulting solution was hydrolysed with resin as described in Experiment 33 and the hydrolysate examined by descending chromatography on Whatman No.1. paper in solvent II, and the sugars located with spray I.

Experiment 35.

Chromatographic investigation of sample C

Sample C was placed in a streak on Whatman No.3. paper. After chromatography in solvent II the sugars were located in strips from each side of the paper with spray I. The two bands behind lactose were cut out and eluted with water. The eluants were hydrolysed with resin as described in Experiment 33 and evaporated almost to dryness under reduced pressure. They were examined by descending chromatography on Whatman No.1. paper in solvents I and II, and the sugars located with spray I. Another chromatogram in solvent II was sprayed with reagent IV in order to locate the sugars.

Experiment 36.

Chromatographic investigation of sample D

Sample D was treated as described in Experiment 35.

Experiment 37.

Electrophoretic examination of sample D

A portion of sample D was treated with potassium borohydride in order to convert the reducing sugars to the corresponding alcohols. Electrophoresis on Whatman No.3. paper was carried out in 0·062M-sodium molybdate buffer pH 5·0 at 21 v/cm. for 45 min. Sugars were located with spray III.

Experiment 38.

Studies on liver specimens from cases of suspected glycogen storage diseases

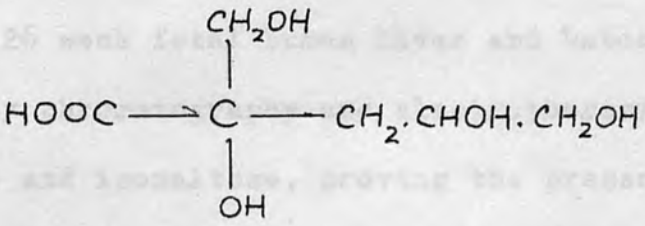
Four liver samples, P.M., D.H., J.H. and S.L., were ground with trichloracetic acid and the glycogen precipitated with ethanol as described in Experiment 2. Average chain lengths of the glycogens were determined by incubation with α-amylase (Experiment 10) and exterior chain lengths were determined by incubation with β-amylase (Experiments 5 and 6). The values of λ_{max} of the glycogen-iodine complex was determined as in Experiment 23.

MAIN SECTION

(a) Studies on glycogens

The readily available supplies of laboratory-bred animals has facilitated research into their fetal metabolism and development. Human tissues, however, can only be obtained from miscarriages or medically terminated pregnancies, and a comprehensive survey of the whole gestation period from any aspect is thus made very difficult. In this study the fetal livers obtained ranged in age from 11½ to 26 weeks. The work was undertaken (i) to provide information on the storage and structure of glycogen in the human fetal liver and (ii) to examine some of the enzymes concerned with glycogen metabolism. In addition to increasing the fundamental knowledge of the developing human liver, such information would have a direct bearing on the glycogen storage diseases in children.

The stability of glycogen to hot concentrated alkali has been questioned by several workers.[270,271,272] Reductions in the molecular weights of glycogens by alkali treatment are not consistently observed, however,[270,273] and Stetten and Katzen[274] have suggested that, provided anaerobic conditions are maintained, only the reducing end of the molecule will be affected. The products are polysaccharide acids and a small amount of free isosaccharinic acid.



Degradation does occur if hot dilute alkali is used as the extractive,[270,275] as oxygen is more soluble in the dilute solution and anaerobic conditions no longer prevail.[276]

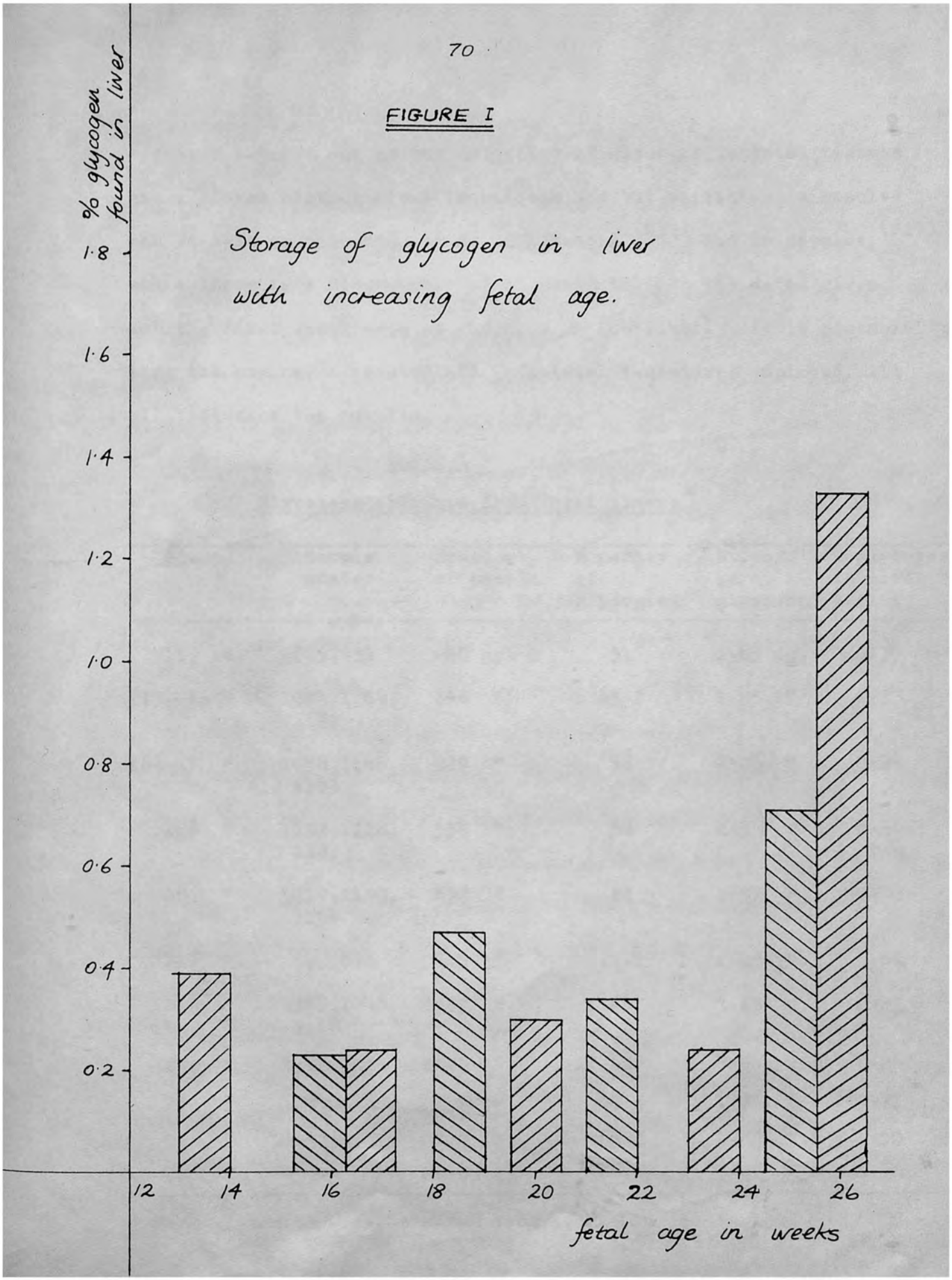
Trichloracetic acid is generally considered to be less effective as an extractive [277] unless adequate homogenisation is used [278] or the homogenate heated to 100° for 15 minutes.[279] In the extraction of the fetal glycogens (Table I) trichloracetic acid was used, as previous extractions of human and other mammalian liver glycogens had resulted in higher yields by this method than by the use of alkali. The samples were also purer, and contained only trace amounts of nitrogen. In the case of many fetal glycogens it was important that fairly pure samples should be obtained initially, since further purification of the 1-5 mg. samples obtained would have resulted in the loss of the sample.

Fetal livers aged 11½, 12, 13-13½, and 14½-15 weeks gave only a trace of precipitate when extracted with trichloracetic acid. Partial acid hydrolysis of these precipitates (Experiment 16), followed by chromatographic examination, showed that no reducing sugars were present, whereas the two control hydrolysates (glycogen

from a 26 week fetal human liver and baboon liver glycogen) were shown by chromatography and electrophoresis to contain glucose, maltose and isomaltose, proving the presence of α-(1→4)- and α-(1→6)- linkages in the original molecule. Milligram quantities of glycogen have been obtained from fetal livers ranging from 13 to 26 weeks of age (Table I). Kitamura (234) found that deposition of glycogen in the human fetal liver began at 17 weeks, $3\frac{1}{2}$ weeks later than in this study, although the substantial increase in storage of liver glycogen beginning at 26 weeks (Fig.I) corresponds very closely to his observations. The increase in glycogen storage is not steady, but can be accounted for by normal biological variation.

Small quantities of glycogen have also been found in the human liver as early as 6 weeks by Shapovalov (229) who used histochemical techniques to identify the polysaccharide. In support of this, Mackay and his coworkers have suggested that the 4 week-old fetal liver is almost ready to assume the function of glycogen storage.(232) The chemical methods that have been employed in the present study do not appear to have been sensitive enough to reveal such small quantities of glycogen; not until the 13th week of gestation is glycogen detectable by chemical means. The report by Lelong and coworkers (235) that they could find no glycogen in the fetal liver until a few weeks before term is difficult to reconcile with other observations. The evidence for the presence of glycogen outweighs this single latter report. Most of the

FIGURE I


Storage of glycogen in liver with increasing fetal age.
% glycogen found in liver
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
12
14
16
18
20
22
24
26
fetal age in weeks

research carried out on the detection of glycogen in fetal tissues has employed histochemical techniques and the variation in results can be seen by comparing those of Kitamura (234) and Shapovalov, (229) where there is a discrepancy of 11 weeks between the dates given for the first appearance of glycogen in the liver. It is probable that the stains, apparatus and individual techniques employed will all influence the results.

Table I

Glycogens obtained from fetal livers *

Age	Sample number	Fresh wt. of sample	% purity of glycogen	Wt. of pure glycogen	% glycogen in liver tissue
13½ wk.	895,935	420 mg.	51	1·65 mg.	0·39
15½-16 "	1074,1164, 1172	846 "	45·2	1·95 "	0·23
16½-17 "	1058,1148, 1183	929 "	58	2·26 "	0·24
18½ "	1152,1128, 1133	554 "	58	2·55 "	0·46
20 "	1117,1190, 1184	870 "	22	2·57 "	0·30
21½ "	893,894	465 "	27·2	2·23 "	0·48
21½ "	1167,1048, 1168	1567 "	68	3·13 "	0·20
23½ "	1182,1084	1944 "	60	4·68 "	0·24
25 "	850	246 "	83·5	1·75 "	0·71
26 "	926	180 "	66·3	3·05 "	1·70
26 "	-	28·83 g.	86.6	320 "	0·96

* Most glycogens were obtained from pooled fetal tissues.

In all mammalian species studied so far, there is a marked increase in glycogen deposition at a fairly late stage of gestation. From the figures quoted [223,239,241] it appears that in rats and guinea-pigs the onset of glycogen deposition occurs after about 85% of the total gestation period has elapsed. On this basis no marked increase in glycogen storage should be noted in the human fetus until approximately the 33rd week of gestation. However, from observations made on lambs, monkeys,[240] rabbits [280] and pigs,[281] it would appear that the stage at which glycogen accumulation increases rapidly is related to the class of mammal. In rats, guinea-pigs and rabbits (rodents) it occurs fairly late in gestation, after 90%,[229] 80%[239] and 75%,[280] respectively, of the gestation period has elapsed. In man (primate) it occurs after 66% of the gestation period has elapsed, while in Rhesus monkeys glycogen stores are very high at 113 days (67% of gestation).[240] Unfortunately no fetuses younger than 113 days were studied, so it is not known exactly when glycogen storage begins its increase. Pigs and sheep (ungulates) vary rather widely, the increase occurring after 88% of gestation in the former [281] and 61% in the latter.[240]

The factors responsible for the storage of glycogen in the liver are largely hormonal. Adrenal and pituitary hormones[222,223] and insulin [218-221] are known to stimulate glycogen synthesis and it is interesting to note that in fetal lambs the appearance of the islets of Langerhans (the source of insulin) coincides with

the accumulation of glycogen in the fetal liver.[240] Thus it would seem that the fetal hormonal systems of the primates and, to a lesser degree, of the ungulates, mature at a far earlier stage of gestation than those of the rodents.

The deposition of glycogen in tissues other than hepatic has been observed at very early stages of gestation.[229,230,235] It is generally accepted that there is no hormonal control over these glycogen stores, and their formation is initiated most probably when the enzyme system for glycogen synthesis and breakdown is completed in the particular organs. Glycogen supplies in these areas normally decrease as gestation advances and are very low at term.

The late appearance of the enzyme glucose-6-phosphatase and subsequent depletion of glycogen stores has been noted in several mammalian fetuses. The function of this enzyme is presumably to provide an abundant supply of blood glucose, in order that the fetus will have sufficient energy to survive birth and the first few days of neonatal life. There have been no reports of the same pattern in human fetuses, although indirect evidence obtained by Kitamura [234] indicated that hepatic glycogen decreased rapidly at birth. It is also known that the level of blood glucose is high in new-born infants.[282,283] Glucose-6-phosphatase activities determined on 8 fetuses ranging in age from 13 to 23 weeks (Experiment 27) showed that either the enzyme was completely absent, or that its activity was so low as to require a more

sensitive method for its determination. It was very unlikely that the enzyme had been inactivated by leaving the liver specimen too long either at room temperature or at -15°, since portions of mouse liver were found to possess fairly constant activity when left at room temperature for up to 3 hr. and at -15° for up to 6 months. Very low activities have been reported by Cori and Schulman (284) for an 18 week-old human fetus, and by Auricchio and Rigillo.(242) The latter workers examined human fetuses in the 5-9 month range and could not detect any sudden increase in activity. If this does occur, it is presumably very close to or just after term. The report by Villee (227) of a fully active enzyme at 20 to 24 weeks of gestation was based on the production of glucose by slices of fetal liver. This method does not exclude the possibility that the glucose arises from the α-amylase and α-glucosidase pathways from the liver glycogen. This could well explain the high activities which are not in agreement with those determined by the release of inorganic phosphate from glucose-6-phosphate. Villee himself suggests that, taking into account the variation in the activities recorded, the appearance of the enzyme may sometimes be delayed until birth or after.

Human fetal liver is of great value to the medical world as it possesses the capacity of producing red blood cells (erythropoiesis). Owing to this, the demand for the liver tissue is high and many of the samples obtained for extraction of glycogen

were of the order of 1-5 mg. glycogen (Table I). To determine the structure of these glycogens, the micro-methods employing the enzymes β-amylase and α-amylase (Experiments 6 and 10) were adopted. When tested on glycogens of known structure, each method gave results in good agreement with those determined by periodate oxidation (Experiment 11) and β-amylolysis using the Somogyi reagent (257) to measure the maltose produced (Experiment 5). The α-amylase preparation (Experiment 7) was found to have only a slow hydrolytic action on maltose (Experiment 8). Glucose production after 12 hr. was barely discernible, while after 24 hr. it was far more marked. The incubation time of 15 hr. was therefore chosen as being convenient and liable to produce no more than trace amounts of glucose from the action of the maltase.

The structure of the fetal glycogens (Table II) obtained by these methods show them to be within the range of variation encountered by previous workers (Table III). The average chain lengths vary from 12·5 to 14·5 D-glucose units and the average exterior chain lengths from 7·7 to 10·2 D-glucose units. The structures of the glycogens from the 18½ week-old and 21½ week-old (1167, 1048, 1168) fetuses indicate that the outer chains are a little shorter (1-2 D-glucose units) than normal. It is doubtful whether this finding has any significance, as the methods employed have an error of ± one D-glucose unit. (86)

TABLE II

Structures of fetal glycogens

Age	Sample number	$\overline{CL}$ by α-amylase	$\overline{ECL}$ by β-amylase	$\therefore \overline{ICL}$ †
13½ wk.	895,935	14·1	9·2	3·9
15½-16 "	1074,1164, 1172	13·0	10·2	1·8
16½-17 "	1058,1148 1183	13·5	8·7	3·8
18½ "	1152,1128, 1133	12·5	7·7	3·8
20 "	1117,1190, 1184	14·5	-	-
21½ "	893,894	13·7	9·1	3·6
21½ "	1167,1048, 1168	13·6	8·0	4·6
23½ "	1182,1084	14·1	9·0	4·0
25 "	850	14·0	8·7	4·3
26 "	926	13·5	8·4	4·1
26 "	-	13·9*	8·9	4·0

† $\overline{ICL} = \overline{CL} - \overline{ECL} - 1$

* $\overline{CL}$ by periodate oxidation = 13·4

TABLE III

Structures of other human and mammalian glycogens †

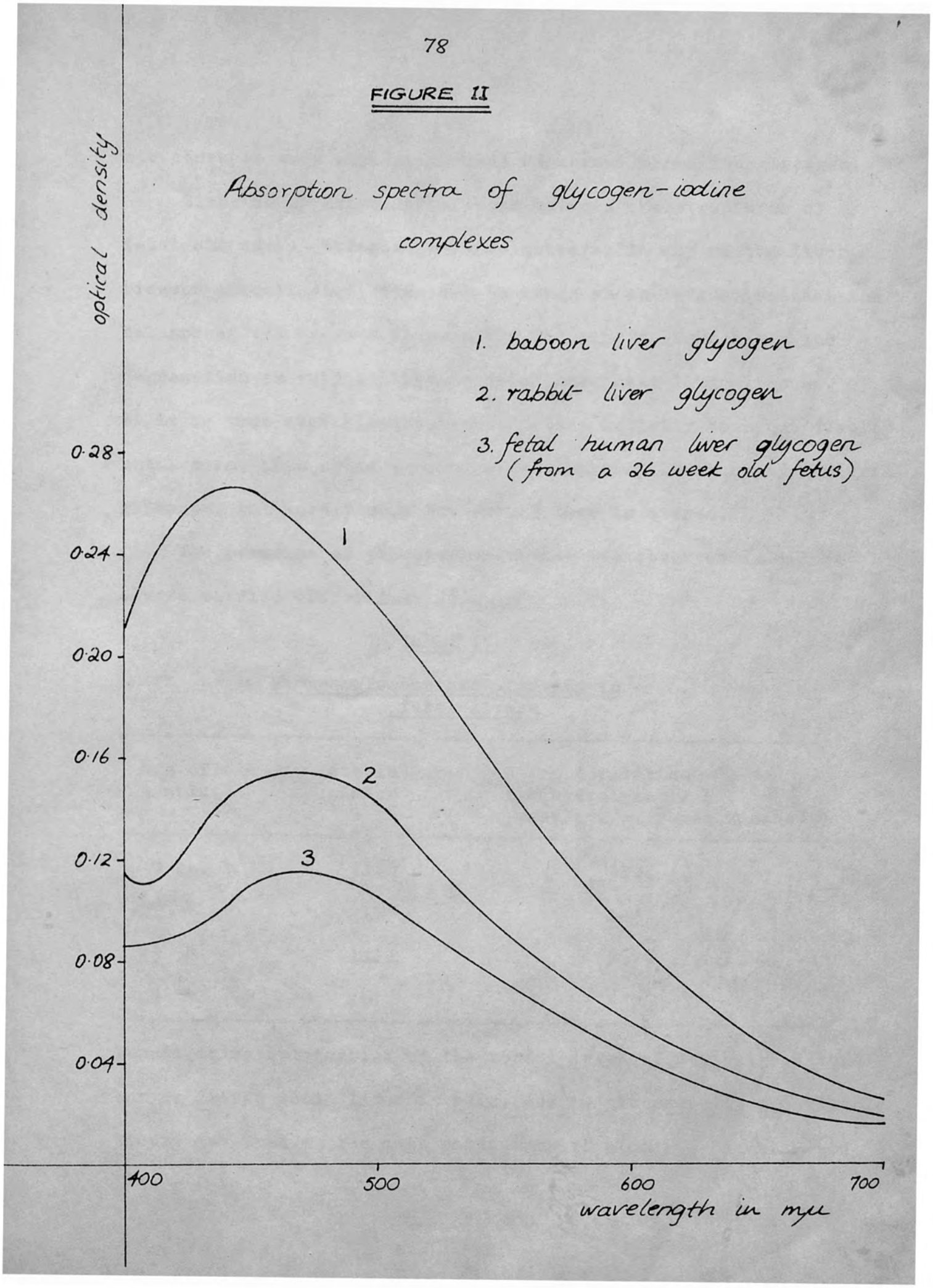
Sample	$\overline{CL}$	$\overline{ECL}$	$\overline{ICL}$
*Human liver	14·0	8-9	5-6
Human muscle	11	7	3
Rabbit liver IV	13	8-9	3-4
Rabbit liver V	14	9-10	3-4
Rabbit muscle II	11	6-7	3-4
Cat liver IV	13	9-10	2-3
Fetal pig liver	11	8	2
Fetal sheep liver	13	9	3
Horse liver	11	-	-
Ox liver	12-13	-	-

† values taken from reference 25.

* from a case of glycogen storage disease.

Glycogen from the 23½ and 26 week-old fetuses gave glycogen values with concanavalin A of 0·82 and 0·89, respectively, (Experiment 25), which, although the lowest values in this study, were within the range of variation 0·76 to 1·36 found by Manners and Wright.(42) Glycogen from the 26 week-old fetus formed a reddish-brown complex with iodine, the absorption maximum being at 470 mμ (Figure II). Both these latter results support the finding that fetal glycogen

FIGURE II


Absorption spectra of glycogen-iodine complexes
1. baboon liver glycogen
2. rabbit liver glycogen
3. fetal human liver glycogen (from a 26 week old fetus)
optical density
0·28
0·24
0·20
0·16
0·12
0·08
0·04
1
2
3
400
500
600
700
wavelength in mμ

structure is very similar to that of normal mammalian glycogen.

Since no striking differences between the structures of fetal and adult glycogens were encountered in any of the liver tissues investigated, this can be taken as an indication that the balance of the enzymes responsible for glycogen synthesis and degradation is very similar in fetal and adult liver tissues. If it is true that glucose-6-phosphatase activity does not develop until term, this would have no effect on the structure of the fetal glycogen, but merely upon the amount that is stored.

The presence of phosphoglucomutase was observed in all fetal livers studied (Experiment 28).

TABLE IV

Phosphoglucomutase activity in fetal livers

Age of sample	Sample number	Activities (μM hydrolysable P used/hr./g.frozen tissue)
9 wk.	1180	3108
21 "	1175	1437
23 "	1125	2679
24 "	1018	2326

Quantitative information on the normal level of activity of this enzyme in the human liver is rare, due to the scarcity of suitable biopsy material. The most comprehensive study is by Weber and

Cantero,[285] who give a value of 10149 ± 5046 μM/hr./g. wet wt. for human liver. Calderbank and coworkers[11] give values of 5088, 1536, and 1704 μM/hr./g. frozen tissue, when the original liver homogenate is diluted 10-, 100- and 500-fold. The values in Table IV correspond more closely to Calderbank's results, although such high dilutions were not employed. Taking the results of Weber and Cantero as being more representative of normal values at lower dilutions, the fetal liver enzyme is rather low in activity. Nevertheless, its presence cannot be denied. Weber and Cantero also give figures for the activity of phosphoglucoisomerase in the human liver. Their value of 15630 ± 5241 μM fructose-6-phosphate/hr./g. wet wt., i.e. approximately 250 units/g. wet wt. is five times higher than those found in the present study (Experiment 29).

TABLE V

Phosphoglucoisomerase activity in fetal livers

Age	Sample number	Activity (units/g. frozen tissue) *
13 wk.	1162	56
14½ "	1009	52
16 "	1172	51
17 "	940	61
22 "	1168	48

* One unit = the quantity of enzyme which produces 1 μM fructose-6-phosphate per minute at 37°.

Table V shows that the enzyme is present at 13 weeks, and maintains a fairly constant level up to 22 weeks. The fetal liver has been shown to possess a high rate of glycolysis,[225] which requires adequate activities of phosphoglucomutase and phosphoglucoisomerase. From the data in the present study, neither of these enzymes show activities as high as those encountered in the normal adult human. They may, however, be adequate to maintain a fairly high rate of glycolysis. Neither enzyme shows any change in activity with advancing fetal age in the period studied, although it may be surmised that a rise in activity will occur in both enzymes later in gestation, since it has been shown that in fetal rats and guinea-pigs[97,237,243] both enzymes have reached normal adult levels or higher at the end of gestation.

Ten samples of liver from children ranging in age from 6 weeks to 10 years 11 months were obtained and the glycogen extracted by alkali (Experiment 1) or trichloracetic acid (Experiment 2). The yields of glycogen, given in Table VI, varied from 0·43 to 5·6%. The purities of the samples were determined by acid hydrolysis (Experiment 3).

TABLE VI

Glycogens obtained from children

Age	Fresh wt. of sample	% purity of glycogen	Wt. of pure glycogen	% glycogen in liver tissue
† -	3·28 g.	100	14·1 mg.	0·43
12 wk.	1·09 g.	85·3	19·7 "	1·81
8 "	43·6 mg.	57·3	1·95 "	4·5
2yr.4m.	234·9 "	98·6	13·2 "	5·6
1yr.8m.	188·6 "	45	1·4 "	0·74
14 wk.	358·6 "	67·6	4·3 "	1·2
12 "	1·298 g.	69·7	15·9 "	1·2
6 "	50 mg.	45	1·4 "	2·8
10yr.11m.	502 "	76·6	8·4 "	1·67
7 wk.	566 "	62·5	4·5 "	0·8

† Extraction of this sample was by potassium hydroxide.

All other extractions were by trichloracetic acid.

The structures of these glycogens were determined by β-amylolysis (Experiments 5 and 6) and α-amylolysis (Experiment 10). The results (Table VII) show that these glycogens also possess average chain lengths in the normal range of 10-14 D-glucose units, with the exception of the last sample, which has an average chain length of 15·2 D-glucose units. Allowing for the acknowledged error of ± one D-glucose unit in the method employed (Experiment 10) this sample also comes within the normal range.

TABLE VII

Structures of glycogens from children

Age	$\overline{CL}$ by α-amylase	$\overline{ECL}$ by β-amylase	$\therefore \overline{ICL}$
-	13·6	7·9	4·7
12 wk.	13·4	7·9	4·5
8 "	12·6	8·9	2·7
2yr.4m.	12·7	8·6	3·1
1yr.8m.	13·1	9·2	2·9
14 wk.	13·9	9·3	3·6
12 "	12·9	8·3	3·6
6 "	-	†	-
10yr.11m.	12·9	8·2	3·7
7 wk.	15·2	9·2	5·0

† This sample gave 46·5% conversion to maltose.

Five of these samples were treated with concanavalin A (Experiment 25) and their glycogen values measured.

TABLE VIII

Glycogen values determined by the reaction with concanavalin A.

Age	Glycogen value	$\overline{CL}$
2yr.4m.	0·94	12·7
1yr.8m.	1·19	13·1
-	1·03	13·6
12 wk.	0·95	13·4
7 wk.	0·96	15·2

All five values do not differ significantly from unity, indicating that they possess structures typical of normal glycogens.

Baboon liver glycogen was extracted by digestion with potassium hydroxide, and its purity and structure determined as previously described. The value of the average chain length was found to be 13·3 D-glucose units both by periodate oxidation and α-amylolysis. The average exterior chain length was found to be 8·3 D-glucose units, thus giving an average interior chain length of 4 D-glucose units. The complex with iodine (Fig II) was found to have an absorption maximum at 435 mμ. These results indicate that baboon liver glycogen has a normal structure. The results obtained from the reaction with concanavalin A are more difficult to interpret. Baboon liver glycogen has a glycogen value (G.V.) of 1·5, while the β- and glucamylase-limit dextrins have values of 1·74 and 1·12 respectively. The figure obtained for the baboon liver glycogen was by far the highest recorded in this study, although not outside the normal range, since Smith and coworkers [41] have found glycogen values of 1·45 and 3·0 for rabbit hair glycogen and baker's yeast glycogen respectively. The glycogen value of baboon liver glycogen increased to 1·74 on β-amylolysis. This is in accordance with the results of Smith [41] and Manners.[42] However, the glucamylase-limit dextrin, while possessing a similar

structure to the β-limit dextrin, had a lower glycogen value than the normal baboon liver glycogen. At present no explanation can be advanced for this observation, since the precise nature of the reaction between glycogen and concanavalin A is not known, and no anomalies have been shown in the structures of these glycogens.

The structures of mouse and rabbit liver glycogens were determined as described previously. Mouse liver glycogen was found to have a normal structure, its average chain length being 13·8 D-glucose units by α-amylolysis and 13·1 D-glucose units by periodate oxidation. β-amylolysis gave an average exterior chain length of 8·6 D-glucose units, and hence the average interior chain length was 3·5 D-glucose units. Further evidence of a normal structure was provided by the fact that the glycogen value (Experiment 25) was 0·9, and that the iodine complex (Figure II) had an absorption maximum at 455 mμ. Rabbit liver glycogen also had a normal structure, having an average chain length of 12·6 D-glucose units by α-amylolysis and 13·9 D-glucose units by periodate oxidation. The average exterior chain length was found to be 8·1 D-glucose units by β-amylolysis and hence the average interior chain length was 4·2 D-glucose units. The absorption maximum of the iodine complex was 470 mμ (Figure II), which is within the normal range.

(b) Studies on hospital cases

Four samples of human liver were obtained from cases of suspected glycogen storage disease, and their structures determined by the methods described in Experiment 41.

Patient P.M. was subject to fits and his physical development was somewhat retarded. He was found to be hypoglycaemic and, at laparotomy, islet cell hyperplasia of the pancreas was discovered. Glycogen from a sample of liver obtained at laparotomy was found to have a normal structure. The average chain length was found to be 12·5 D-glucose units by α-amylolysis, and β-amylolysis gave a value of 8·2 D-glucose units for the average exterior chain length, and hence an average interior chain length of 3·3 D-glucose units. Later it was found that L-leucine caused the hypoglycaemia. The latter could be controlled by a diet low in L-leucine, thus ruling out the possibility that the hypoglycaemia was caused by glycogen storage disease.

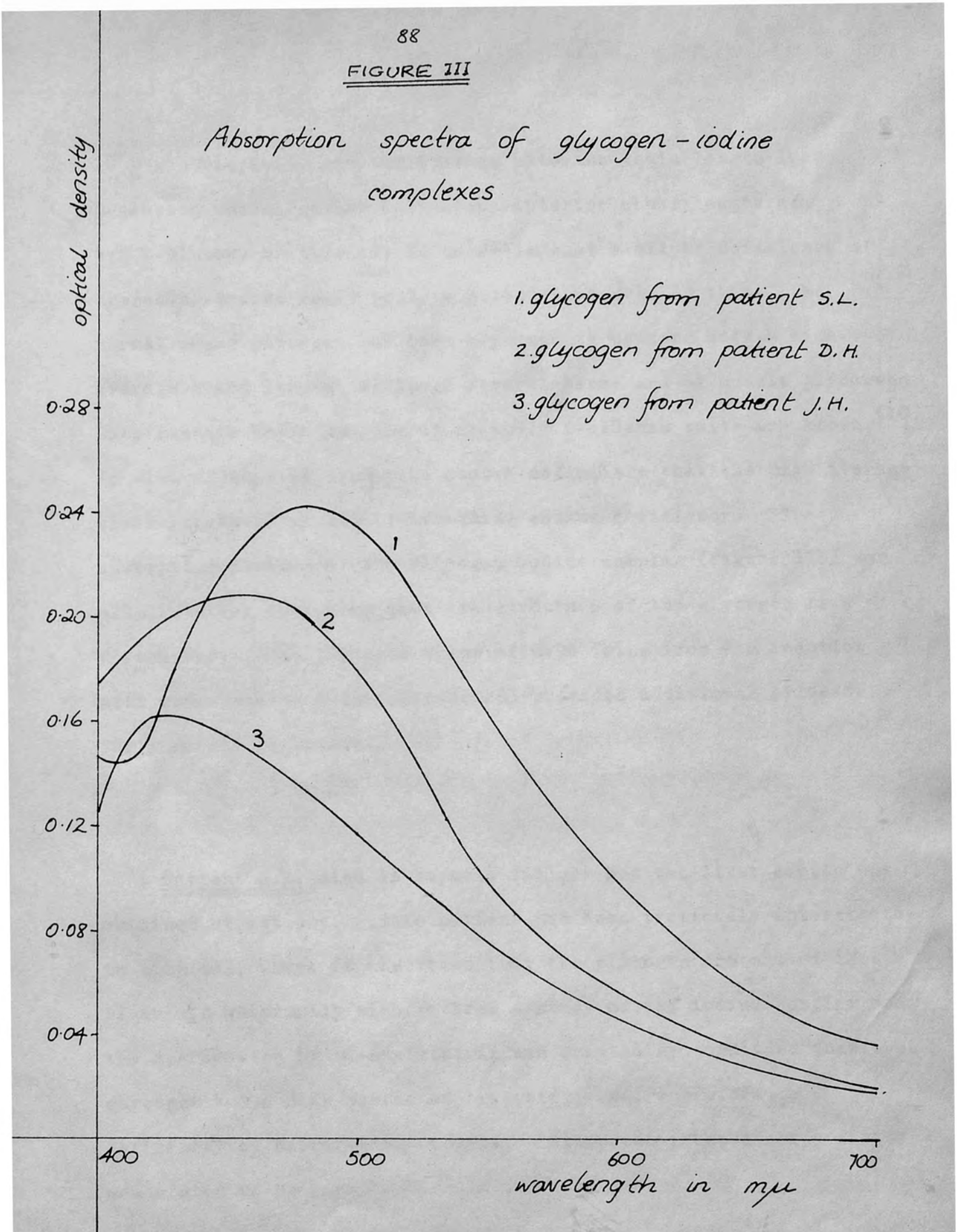
Patient D.H. had an enlarged liver and although at first she was small and underweight, she soon began to put on weight rapidly. A histological examination of the liver had shown some accumulation of glycogen and fat and normal levels of glucose-6-phosphatase and phosphorylase. The response to glucagon was normal, supporting the histological evidence for the presence of glucose-6-phosphatase.

Glycogen storage disease had been suggested as a possible explanation. The glycogen obtained from a biopsy specimen was found to have a structure a little more compact than any that had been encountered in human specimens.[25] The average chain length was 10·5 D-glucose units and the average exterior chain length 6·8 D-glucose units, giving an average interior chain length of 2·7 D-glucose units. The average chain length was 2 D-glucose units lower than other human glycogens. This result may not be significant, since the method employed for the determination of the average chain length is accurate only to ± one D-glucose unit, and the $\overline{ECL}:\overline{ICL}$ ratio was approximately 2:1, indicating normal branching characteristics. Also the absorption maximum of the complex with iodine was 450 mμ (Figure III), which was well within the range normally encountered. As yet, no definite diagnosis has been made on the patient, who remains fairly well.

Patient J.H. was also found to have an enlarged liver. She was hypoglycaemic and had a poor response to adrenaline and D-fructose, but a fairly good response to intravenous injection of glucagon. Histological examination had shown a large accumulation of glycogen and fat, and a low activity of glucose-6-phosphatase and branching enzyme. The structure of the glycogen was found to be a rather open one. The average chain length was

FIGURE III

Absorption spectra of glycogen-iodine complexes



16 D-glucose units and the average exterior chain length 10·8 D-glucose units, giving an average interior chain length of 4·2 D-glucose units. It is possible that a slight deficiency of branching enzyme could produce a structure such as this. No normal human glycogen has been reported to possess such a high average chain length, although several horse and ox muscle glycogens with average chain lengths of up to 17 D-glucose units are known.(10) In view of this it cannot be stated definitely that the high average chain length is caused by branching enzyme deficiency. The absorption maximum of the glycogen-iodine complex (Figure III) was 425mμ, which indicates that the structure of the glycogen is a normal one. The glycogen value of 0·96 found from the reaction with concanavalin A (Experiment 25) provides additional evidence for a normal structure.

Patient S.L. died in hepatic failure and the liver sample was obtained at autopsy. This patient had been previously investigated in hospital, where it was found that the glycogen content of the liver was abnormally high. From a study of the iodine complex and the degradation by α-amylase, it was originally concluded that the glycogen had a high degree of branching, and, therefore, a deficiency of debranching enzyme. Glucose-6-phosphatase activity was stated to be normal.

The present findings are completely at variance with this. The structure of the glycogen appears to be almost identical with that of patient J.H. The average chain length was found to be 16.5 D-glucose units by α-amylolysis and 15.9 D-glucose units by periodate oxidation. The average exterior chain length was found to be 11.0 D-glucose units by β-amylolysis, giving an average interior chain length of 4.2 D-glucose units. As with patient J.H., it is uncertain whether this structure is the result of a slight branching enzyme deficiency. One striking difference between the glycogens from patients J.H. and S.L. occurs in their reaction with iodine. The iodine complex with J.H. glycogen has an absorption maximum at 425$m\mu$, whereas the complex with S.L. glycogen has its absorption maximum at 480$m\mu$, despite the almost identical structures of the two glycogens. Obviously the value of the absorption maximum is not always an infallible guide to the structure of the glycogen.

Patient G.B. was a young baby who failed to gain in weight almost from birth, and was found to be unable to metabolise lactose or sucrose in the intestine. Accordingly milk was omitted from his diet. When arrowroot or rice was included as the carbohydrate source, urine samples were found to contain a reducing sugar very similar to lactose in its behaviour on

chromatography in ethyl acetate: pyridine : water (10:4:3) solvent, although it was not hydrolysed by lactase (β-D-galactoside galactohydrolase).(286) Chromatographic examination of urine samples in solvents I, II, III and IV (Experiment 30) failed to elucidate the nature of the unknown sugar, since its R_F value in all four solvents was identical with that of lactose. The mobilities of the unknown sugar and its reduced form, when subjected to paper electrophoresis (Experiments 31 and 32), corresponded to those of isomaltose and isomaltitol respectively. Separation of the reducing sugar from the urine, followed by hydrolysis, revealed glucose as the only sugar present (Experiment 34). From this evidence it can be concluded that the unknown sugar was isomaltose. The isomaltose probably arises from the α-(1$\rightarrow$6)-linked D-glucose units in amylopectin, which constitutes 81·5% of rice starch and 79·5% of arrowroot. Thus besides lacking the enzymes, which hydrolyse sucrose and lactose, the patient was also appreciably deficient in isomaltase (isomaltose 6-glucohydrolase). When amylose was substituted for arrowroot or rice, thus removing the source of α-(1$\rightarrow$6)-linked D-glucose units, the patient's condition improved. He gained a few pounds in weight and no more isomaltose could be detected in the urine. At this stage a preparation of milk, treated with lactase to remove the lactose, was included in the diet, and the urine samples collected then showed the presence of two sugars with R_F values slightly less than lactose in solvent II

(ethyl acetate : pyridine : water, 10:4:3). The same or similar sugars were found to be present in the lactose-reduced milk.(286) When samples C (lactose-reduced milk) and D (urine taken when the patient was fed on a diet containing lactose-reduced milk and amylose) were examined as described in Experiments 35 to 37, it was found that both the unknown sugars contained glucose and galactose, and from their chromatographic behaviour were most probably disaccharides. The reduced forms streaked badly when examined by paper electrophoresis in molybdate buffer and no accurate deductions could be made about the linkages. However, it is fairly certain that the sugars appearing in the urine are the same as those present in the lactose-reduced milk. They are probably formed by transglycosylation:

$$\text{Gal 1} \xrightarrow{\beta} 4\ \text{Gluc} \rightleftharpoons^{\text{lactase}} \text{Enzyme-Gal} + \text{Gluc}$$

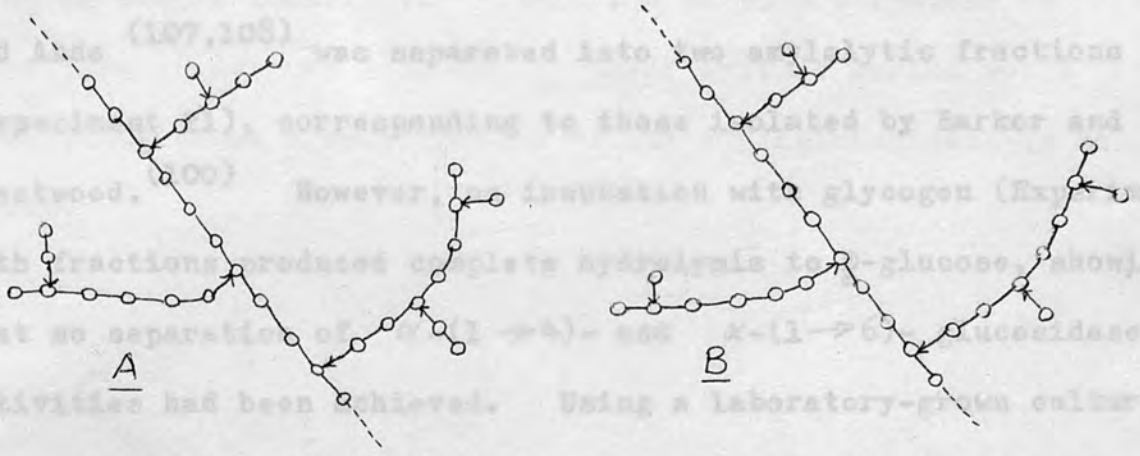
$$\text{Enzyme-Gal} + \text{Gluc} \rightleftharpoons \text{Gal 1} \xrightarrow{?} ?\,\text{Gluc} + \text{Enzyme}$$

The patient is unable to metabolise these new products, indicating the lack of yet another enzyme. The presence of these sugars appear to contribute to the patient's lack of progress and it would be advantageous to find a method of removing them from the milk. This is at present being investigated at Royal Holloway College.

(c) Studies on glucamylase

The action of glucamylase on rabbit liver glycogen was complete after 26 hr. (Experiment 12) and resulted in the production of 54·5% D-glucose. The production of maltose from β-amylase action on rabbit liver glycogen was 46·8%. Assuming that β-amylase leaves stubs on an average length of 2·5 D-glucose units,[82] the figure for the action of glucamylase shows that this enzyme leaves stubs of average length 1·5 D-glucose units. Incubation of rabbit liver glycogen with β-amylase, followed by incubation with glucamylase (Experiment 13) resulted in a final production of D-glucose of 60·2%, indicating that stubs of average length of 1 D-glucose unit were left after glucamylase action. Periodate oxidation (Experiments 16 and 17, Figure IV) of the limit dextrins isolated after β-amylase (Experiment 14) and glucamylase (Experiment 15) action gave average chain lengths of 7·2 D-glucose units for the β-limit dextrin and 6·0 D-glucose units for the glucamylase-limit dextrin. The corresponding values determined by α-amylolysis were 7·5 and 6·5 D-glucose units respectively. From the above data it appears that glucamylase removes an average of one D-glucose unit more than β-amylase, leaving stubs of average chain length 1·5 D-glucose units. These stubs would consist of either single D-glucose units or maltose units, linked to the rest of the molecule by an α-(1→6)-linkage. In Experiment 13 the incubation with glucamylase was a prolonged

one of 5 days. If left for this length of time, the enzyme appears to reduce all the stubs to one D-glucose unit in length, but does not attack the branch linkages.



A - Structure of glucamylase-limit dextrin after 1-2 days' incubation with glucamylase

B - Structure of the dextrin after 5 days' incubation

The amount of degradation caused by the Aspergillus amyloglucosidases varies with the species and even among the same species. Preparations from A. oryzae,[103] for example, effect a 100% conversion to D-glucose, whereas various preparations from A. niger result in 50-60%[101,106] or 100%[107,108] conversions to D-glucose. The complete hydrolysis caused by these enzymes is not due to contamination by α-amylase, since careful steps have been taken to remove this impurity. It appears that most of the pure enzyme preparations are capable of hydrolysing both α-(1 → 4)- and α-(1 → 6)- D-glucosidic linkages, the A. niger preparations by Barker and Fleetwood[100,106] and Kerr

and coworkers [101] being the only exceptions. These workers have prepared enzymes with almost negligible action on α-(1→6)-glucosidic linkages. Using Barker and Fleetwood's chromatographic method for purification, the "Diazyme" preparation used by Pazur and Ando [107,108] was separated into two amylolytic fractions (Experiment 21), corresponding to those isolated by Barker and Fleetwood.[100] However, on incubation with glycogen (Experiment 22), both fractions produced complete hydrolysis to D-glucose, showing that no separation of α-(1→4)- and α-(1→6)- glucosidase activities had been achieved. Using a laboratory-grown culture of *A. niger* NRRL-330-1, the method resulted in the separation of a fraction containing only α-(1→4)- glucosidase activity. From these results it is concluded that the pure α-glucosidase in "Diazyme" is not so specific as that obtainable from *A. niger* NRRL-330-1, prepared by the method of Barker and Fleetwood. The reason for the difference in specificity between the two enzyme preparations is not clear. The *A. niger* strain used in the preparation of "Diazyme" was not specified, neither were the constituents of its culture medium. Both these factors may be important in influencing the enzyme specificity. In this context it is perhaps significant to note that Kerr and coworkers [101] and Barker and Fleetwood [100] used the same strain of *A. niger* and are the only workers who have obtained a preparation with no action on α-(1→6)-linkages.

The inclusion of some compounds into culture media is known to stimulate the production in moulds and bacteria of enzymes capable of utilising these compounds as substrates.[287] This is known as enzyme induction. Inclusion of such compounds as isomaltose or dextran into the A. niger culture medium would conceivably cause a modification of the amyloglucosidase in this manner.

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