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CARBOHYDRATE METABOLISM IN THE LIVER

A Thesis submitted by
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a candidate for the
Degree of Doctor
of
Philosophy

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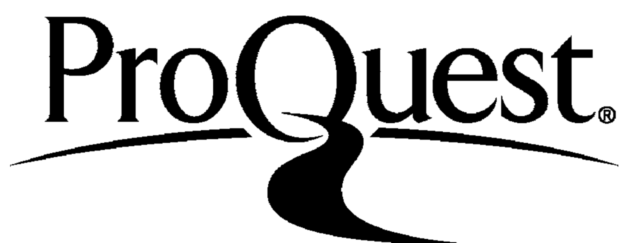

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ABSTRACT

Several human glycogens have been analysed by enzymic methods and shown to have normal structures.

The highly branched structure of rabbit liver glycogen has been verified by stepwise degradation using pullulanase and β-amylase. The actions of pullulanase on the β-amylase and glucamylase limit dextrins of glycogen were also investigated. Iodine complexes formed with rabbit liver glycogen are affected by low molecular weight aliphatic alcohols and by urea. The reaction of the former reagents is important in relation to the purification of glycogens by alcohol precipitation. Some attempt has been made to explain the reaction of urea on the basis of competitive hydrogen bonding.

"Extractable" glycogen was isolated from rat liver by extraction with dilute trichloracetic acid solution. The remaining "residual" glycogen was partially released by proteolytic digestion with Pronase, and isolated by gel filtration on a column of Sephadex G-75. The structures of these two glycogens were found to be similar. The ratio of "extractable" to "residual" glycogen was determined in fetal and adult rat and human liver tissues. The "residual" glycogen content of the developing liver appears to be

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fairly constant but the "extractable" glycogen in the rat, at least, rises to a maximum at term and then falls. These results are discussed on the basis that "residual" glycogen is an artifact and that the level in the liver is governed by the molecular weight of the glycogen and the protein content.

The development of the activities of glycogen synthetase (UDPG:α-1,4-glucan α-4-glucosyltransferase), phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase), phosphoglucomutase (D-glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase) and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase) were examined in fetal and adult human livers, and the activities of these enzymes together with those of α-amylase (α-1,4-glucan 4-glucanohydrolase) and "acid maltase" (α-D-glucoside glucohydrolase) were also measured in fetal and neonatal rat livers. The results were analysed with reference to glycogen storage on the liver.

Glycogen and enzyme analyses have been carried out on the tissues of four suspected cases of glycogen storage disease.

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INTRODUCTION

THE STRUCTURE OF GLYCOGEN

Carbohydrate catabolism is an essential process in all living matter leading to a release of energy which can be utilized for other vital biochemical processes. The nature of the carbohydrate reserve depends on the organism; in higher plants it is commonly starch[1], and in animals, and many lower organisms, glycogen[2].

In animals the polysaccharide, glycogen, is widely distributed in tissues such as brain[3], kidney[4], skeletal and cardiac muscles[5], skin[6] and placenta[7], but the highest concentration occurs in the liver, which acts as a general energy, and hence, glycogen store. The liver also functions as the regulator of the level of "sugar" in the blood, and therefore either degrades or synthesises glycogen in order to maintain this "blood sugar" level[8]. In other tissues, such as muscle, blood glucose is converted to glycogen, but unlike the liver, this glycogen is not catabolised to glucose but is degraded via the glycolytic pathway which releases the large quantities of energy that are necessary for the mechanical function of the tissue.

It was Claude Bernard[9], who, while conducting his physiological experiments on liver function, first isolated glycogen from a dog's liver in 1857. Subsequently,

glycogen was isolated from skeletal muscle[10], placental tissue[11], human liver[12], yeast[13], and several other sources. The early workers described glycogen as a white amorphous powder, producing an opalescent solution in water with a dextro-rotation of 190-200°; addition of iodine to the solution gave a characteristic red-brown colour[14]. The glycogen was found to be unattacked by alkali but was hydrolysed by acid. The product of this hydrolysis was identified as glucose by Külz and Börntrager[15] in 1881, who concluded that glycogen consisted entirely of glucose. Two years earlier Seegan[16] had noted that glycogen was degraded 60-70 per cent by the diastatic enzymes present in saliva and pancreatic juice, to a reducing sugar, later identified as maltose[17]; at that time no significance was attached to this discovery. Further investigations to elucidate the structure of glycogen were hampered until the establishment of the cyclic structure for glucose[18] and the development of the methylation technique for structural analysis[19].

In 1929 Haworth, Hirst and Webb[19] found that glycogen could be acetylated and then regenerated by deacetylation. Furthermore, simultaneous deacetylation and methylation followed by further methylation produced a methylated glycogen which, on hydrolysis, gave 2,3,6-tri-O-methyl D-glucopyranose in 76% yield. This indicated

that the glycogen contained glucose units linked together through the hydroxyl groups at C-1 and C-4.

(i) methylation
(ii) hydrolysis

Glycogen

2,3,6-tri-O-methyl-D-glucose

Confirmation of this linkage was obtained by degradation of the methylated glycogen using acetylbromide, and from the reaction products a disaccharide was isolated, which, after oxidation and methylation, was identified as methyl octa-O-methyl maltobionate[20].

1) methylation
2) acetylbromide
3) removal of bromine

1) oxidation
2) methylation

Methyl octa-O-methyl maltobionate

On hydrolysis, this yielded 2,3,4,6-tetra-O-methyl-D-glucose and 2,3,5,6-tetra-O-methyl-γ-gluconolactone. Thus the presence of α-1,4-linkages between the D-glucopyranose residues in the polysaccharide was established.

In 1932 Haworth and Percival[21] isolated 2,3,4,6-tetra-O-methyl glucose in 8.9% yield from the methanolysis of methylated glycogen, and suggested that this was produced from the terminal end group of a chain; there being 12 glucose units in every chain. As this fact did not agree with the negligible reducing power of glycogen, it was suggested that during the alkaline extraction procedure the reducing end group of this chain had been modified. Further investigations[22,23,24] on various samples of glycogen led to the conclusion that glycogen consisted of chains of either 12 or 18 1,4-linked D-glucopyranose units[25].

Bell[25] in 1936 had carried out an end group assay on a sample of Mytilis glycogen obtaining three products, 2,3,4,6-tetra-O-methyl glucose, 2,3,6-tri-O-methyl glucose and "dimethyl glucoses" in the molar ratio of 1:15:2. This corresponded to an 18 unit chain but the significance of the yield of "dimethyl glucoses" was not realised.

At about the same time the first molecular weight determinations were carried out on glycogen using osmotic pressure measurements. From these Carter and Record[26] estimated that glycogen contained between 3,000 and 5,000

glucose units, and therefore had a molecular weight of approximately 10 million.

On the basis of this evidence, and the fact that methylation of glycogen yielded about 10% 2,3-O-dimethyl glucose, Haworth, Hirst and Isherwood[27] proposed that glycogen had a "laminated" structure comprised of α-1,4-linked D-glucose chains joined to each other at α-1,6-branch points, as follows:

"LAMINATED" STRUCTURE

Terminal end unit

Branch point glucose unit

α-1,4-linked glucose unit

α-1,4-linkage

α-1,6-linkage

The formation of the various methylated products was explained thus:

Methylation

Hydrolysis

2,3,4,6-tetra-O-methyl D-glucose

Hydrolysis

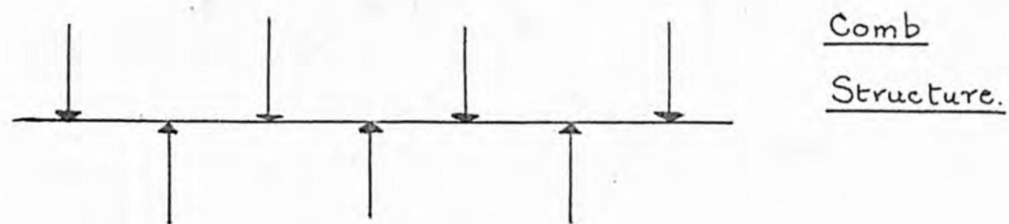
Hydrolysis

2,3,6-tri-O-methyl and 2,3-di-O-methyl D-glucose

The "laminated" structure also supplied an explanation for the lack of reducing power of glycogen.

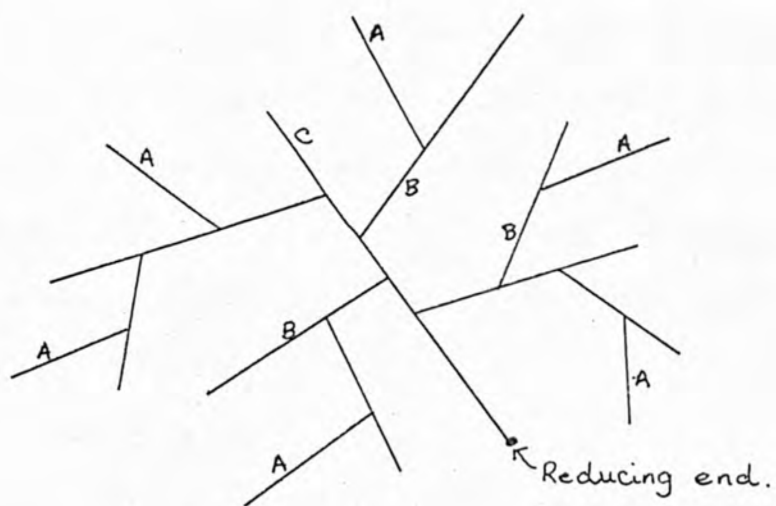
In the same year measurements of the viscosities of several glycogens with molecular weights $\sim$100,000 were found to be similar suggesting that the molecules were spherical[28]. On the basis of this hypothesis (later

shown to be invalid for glycogen[29] which has a molecular weight of 1-100 million), and the evidence from methylation, Staudinger and Husemann[28] proposed the "Comb" structure for glycogen i.e. that glycogen was built up of a long chain of up to 100 α-1,4-linked D-glucose units to which were attached short side chains of 12 or 18 D-glucose residues joined at the C-2, C-3 or C-6 positions of the long chain units.



The technique of β-amylolysis was then used to provide further evidence for the structure of glycogen since it was known that β-amylase, by stepwise hydrolysis, cleaved maltose units from the non-reducing end of an α-1,4-linked D-glucose chain[30]. Meyer and Fuld[31] incubated a sample of mussel glycogen with β-amylase and found that 47% of the glycogen was converted into maltose. The determination of the chain length by methylation assay showed that the chain length of the glycogen was 11 D-glucose units, while that of the β-amylase limit dextrin was 5.5 units long. Meyer therefore postulated that glycogen had a highly branched structure with

terminal residues of 6-7 D-glucose units linked to other chains, with 3 D-glucose units between the branch points; that is a "tree-like" structure.



A=unbranched, B=branched chain. C=one chain per molecule with a free reducing group.

Two years later Meyer and Bernfeld[32] provided additional evidence for the multibranched structure of glycogen by stepwise enzymic degradation using β-amylase and an enzyme preparation from yeast which contained an "isomaltase". On incubating glycogen with the β-amylase a limit dextrin was produced. Incubation of this limit dextrin with the "isomaltase" rendered the dextrin susceptible to further degradation by the β-amylase to give a second limit dextrin. Both the limit dextrins gave colours with iodine solution.

As neither of these enzyme preparations were pure this degradative procedure was later repeated by Larner and co-workers[33] using highly purified muscle phosphorylase and amylo-1,6-glucosidase. After four sucessive incubations of rabbit muscle glycogen with phosphorylase and amylo-1,6-glucosidase, 13% of the original glycogen remained. Comparing this experimental data with the three theories for the structure of glycogen, it was obvious that only the Meyer model could be degraded in the same way, and thus the structure of glycogen was conclusively proved.

Other evidence for the branched structure of glycogen was obtained using various chemical methods. One of these was the oxidation of the polysaccharide by sodium or potassium metaperiodate, which cleaves 1,2-diols. When a glycogen with a chain length of 12 D-glucose units was treated with periodate 1 molecule of formic acid was produced from each terminal glucose unit and 13 molecules of periodate were consumed during the oxidation. Halsall, Hirst and Jones[34] measured the amount of formic acid liberated during the oxidation of several glycogens, thereby determining the average chain lengths, and these results were in good agreement with those estimated by methylation end-group assays.

Abdel-Akher et al. extended this technique by

reducing the periodate oxidised glycogen before hydrolysis. By examining the alcohols produced they[35] were able to determine the chain length of polysaccharides and obtain information about the glycosidic bonds. With glycogen the ratio of glycerol to erythritol was found to be 1:10, in good agreement with the expected value of 1:11 which was based on methylation end group assay[35]. Gibbons and Boissonnas[36] however, found glucose to be present after periodate oxidation, reduction and hydrolysis. This led to the suggestion that glycogen contained a small percentage of anomalous 1,3 or 1,2-linkages, but Bell and Manners[37] were unable to substantiate these results and suggested that the presence of glucose was due to incomplete oxidation.

Erythritol   Glycerol   Glycollic aldehyde

Another technique employed in structural elucidation is that of partial acid hydrolysis using mild conditions so that the polysaccharide is not completely degraded, and the resulting oligosaccharides which are liberated give information concerning the linkages present in the polysaccharide. Wolfrom et al.[38] partially hydrolysed glycogen, acetylated the mixture of products and isolated isomaltose β-octa-acetate. As this was not produced in a control reaction using amylose, it was concluded that the isomaltose represented the branch points in the glycogen. The other products of partial hydrolysis were identified as D-glucose, maltose, isomaltose, and panose confirming the presence of both α-1,4 and α-1,6-links in glycogen[39]. In later investigations Wolfrom and Thompson[40] isolated isomaltotriose and nigerose from the hydrolysate of liver glycogen suggesting the presence of adjacent α-1,6-linkages in the molecule, and a small amount of α-1,3-linkages. The chemical evidence for this is not believed to be conclusive[41].

The advent of infrared spectroscopic analysis[42] provided further proof of the structure of glycogen, as the region 700-1,000 cm^{-1} was found to contain three characteristic absorption peaks; that at 838 cm^{-1} being given by all carbohydrates containing the α-glucosidic linkages, while the peaks at 928 and 760 cm^{-1} indicated that glycogen contained 1,4-linked D-glucose units.

The enzyme α-amylase, which is present in saliva, has also been used to study glycogen structure as it produces 60-70% hydrolysis, as observed by Seegan[16]. The products of this random hydrolysis were identified as maltose, maltotriose and a series of α-limit dextrins containing α-1,6-linkages[43]. Incubation of these α-dextrins with R-enzyme resulted in the liberation of reducing sugars by scission of the α-1,6-links, and the measurement of the amount of sugar produced enabled the percentage α-1,6-links and hence the average chain length to be calculated[44]. It was found however that if high concentrations of α-amylase were used the maltotriose and the dextrins were further degraded liberating glucose[45]. Under these conditions a relationship between the amount of reducing sugar produced and the average chain length was devised[46],

$$\frac{100}{\overline{CL}} = 23.3 - 0.21 \times P_M$$

where $\overline{CL}$ is the average chain length and P_M is the percentage reducing sugar.

Combining this technique with the estimation of the percentage β-amylolysis enabled the average interior and exterior chain lengths to be determined[47].

Recent investigations on the α-limit dextrins have shown that some contain two α-1,6-branch points, separated

by less than three glucose units suggesting that glycogen contains areas of multiple branching[48].

Other evidence of multiple branching was obtained by Liddle and Manners[49] who calculated the amount of this for several specimens of glycogen. The ratio of A:B chains ranged from 1:0.9 - 1:2.9 showing that the value varied depending on the source of the glycogen.

Glycogen was also found to react specifically with a protein extracted from Jack beans, namely Concanavalin A[50], and on measuring the turbidity of a solution of glycogen and Concanavalin A[51] an approximately linear relationship was observed between the degree of branching and the turbidity of the solution for a particular glycogen[52].

Two other α-1,4-glucans exist in addition to glycogen and these are found associated together in plants as starch. One component, amylose, consists of long chains of α-1,4-linked D-glucose units, while the other, amylopectin, resembles glycogen except that the chain length is 20-25 D-glucose units, so that the molecule has a more open "tree-like" structure. All these glucans react to give characteristic colours with aqueous iodine solution, glycogen giving a reddish brown, amylopectin a purple and amylose a deep blue colour; there is a relationship between the colour and the chain length[53].

Investigation showed that iodine forms an inclusion compound with amylose in which six α-1,4-linked glucose units in a helix are required for every iodine molecule[54]. Glycogen with an external chain length of 6-9 units can therefore also form this inclusion compound with iodine but not to the same extent as amylose and amylopectin, and this low binding power has been demonstrated using potentiometric titration methods[55].

The shape of the glycogen molecule is a property which, like so many others, depends on the source of the glycogen. Although it was originally believed that the molecule was spherical[28], recent experiments suggest that in fact it is ellipsoidal[56,57].

X-ray analysis of glycogen gave a diffuse pattern indicative of an amorphous compound[58], and on further investigation it was found that glycogen existed in clusters or rosettes of 60-200 $m\mu$ diameter composed of subparticles of 20-40 $m\mu$ diameter[59,60]. Glycogen in this form has also been synthesised in vitro[61].

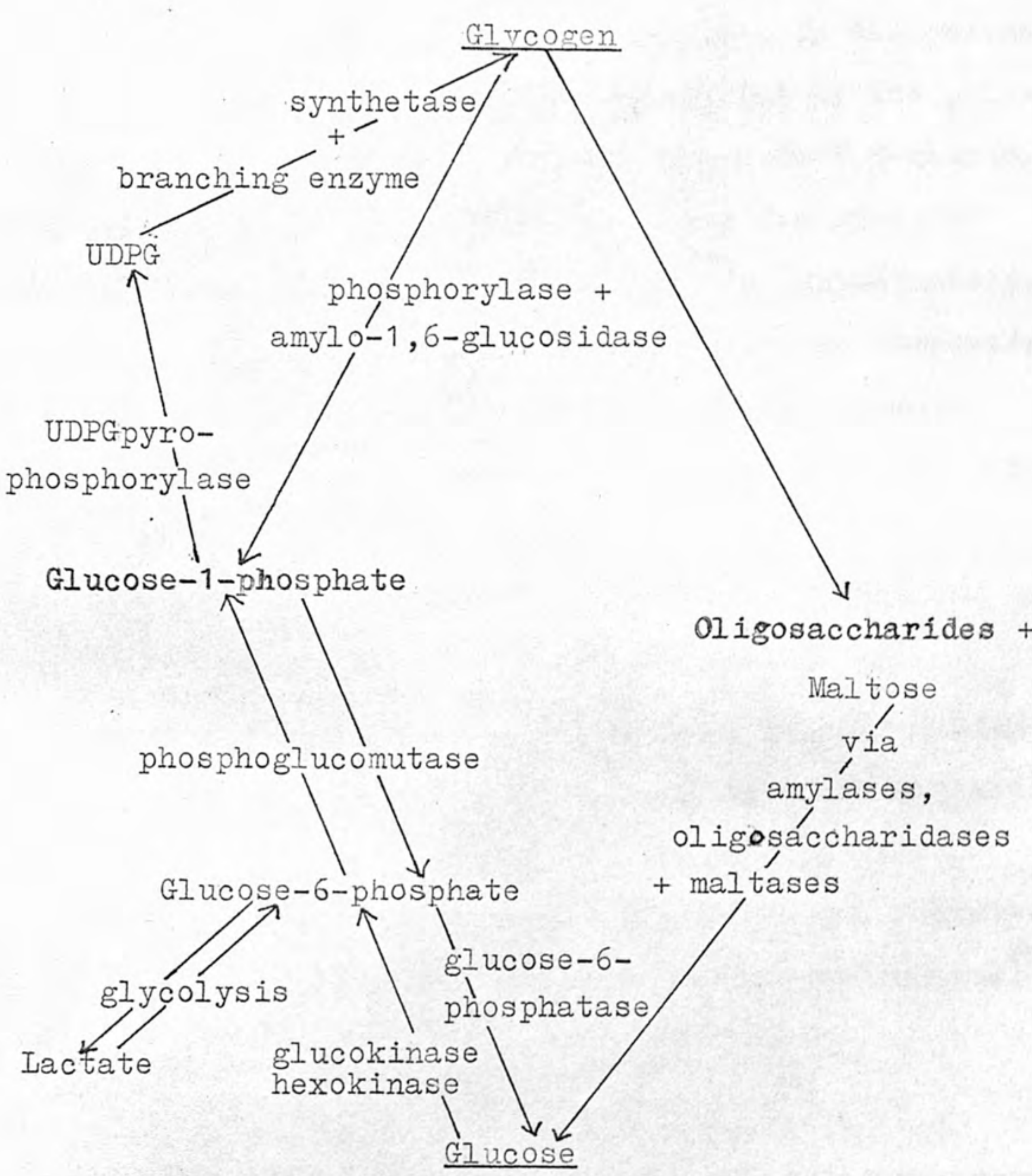
A property depending on the source and also the method of extraction, is the molecular weight[62]. Glycogen, being soluble in water, can be extracted using hot[63] or cold water[64], hot alkali[65], or trichloracetic acid solution[66], and the molecular weight can be determined using osmotic pressure measurements[67] or by light scattering[68] or sedimentation methods[69].

Determinations carried out on glycogen extracted by hot water or alkali gave molecular weight values of $2\text{-}5 \times 10^6$ [63,67] while trichloracetic acid-extracted glycogen had a molecular weight of $11\text{-}80 \times 10^6$ [68], although both alkali and acid were found to degrade glycogen[68]. Analysis by Bell using ultracentrifuge data indicated that glycogen contained two polydisperse components of sedimentation coefficients 60-100S and 150-300S[69]. Similar studies on Ascaris muscle glycogen[62] showed that a cold water extract contained two fractions of molecular weight 450×10^6 and 50×10^6 but extraction by any other methods degraded the glycogen.

Interest, however, has centred on the state of glycogen in the tissues, as it was observed even by early workers that the glycogen appeared to be present in two forms[70]. One form can be extracted with water or trichloracetic acid solution under mild conditions, while the other is only removed by more drastic means such as hot alkali[71]. This difference suggested that the alkali-extracted glycogen was possibly bound to protein; it was known, for example, that glycogen associated with phosphorylase[72]. However when animal tissues were subjected to changes in nutrition or were treated with hormones it was found that the extractable glycogen was more affected than the residual glycogen[73].

Figure I

Pathways of Glycogen Metabolism



ENZYMES OF GLYCOGEN METABOLISM

While the investigations into the structure of glycogen were being carried out, interest in the enzymes concerned in the synthesis and degradation of the polysaccharide was initiated. Phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase) was the first of these enzymes, found by Cori in 1937[74]. Subsequently, numerous other enzymes were isolated, but the discovery in 1957 of synthetase (UDPglucose:α-1,4-glucan α-4-glucosyltransferase) demonstrated the role of nucleotides in the synthesis of glycogen[75].

The postulated pathways for Glycogen metabolism in the body tissues are illustrated in Fig.1.

When a dialysed muscle extract was incubated with glycogen, α-D-glucose-1-phosphate was released indicating the presence of an enzyme, phosphorylase[74]. The reversibility of action of this enzyme was demonstrated by synthesising glycogen from α-D-glucose-1-phosphate,[76]

$$nG\text{-}1\text{-}P \rightleftharpoons G_n + nP_i$$

and both Mg^{2+} ions and adenylic acid activated the enzyme.[77] Bear and Cori[58] investigated the polysaccharide formed *in vitro* by various tissue extracts and found that heart and liver phosphorylase synthesised a glycogen-type

polysaccharide, but that synthesised by muscle phosphorylase resembled plant starch, giving a blue colour with iodine.

The detailed biochemistry of phosphorylase began to be elucidated when Green and Cori, in 1943, isolated crystalline rabbit muscle phosphorylase[78] of molecular weight 340,000-400,000, which was active without the addition of adenylic acid, though this did stimulate the activity. A second, less soluble enzyme was also isolated which differed from the first by being inactive unless adenylic acid was added[79]. These two forms of enzyme were designated phosphorylase a and b respectively. A third enzyme, phosphorylase phosphatase (phosphorylase phosphohydrolase), also present in muscle tissue, was required to interconvert a and b[79]. Studies using P^{32} suggested that this enzyme deactivated phosphorylase a by removing four phosphate groups[80], and, at the same time, releasing two molecules of phosphorylase b from every molecule of phosphorylase a.

$$\text{Phosphorylase } a \rightarrow 2 \text{ Phosphorylase } b + 4\ P_i$$

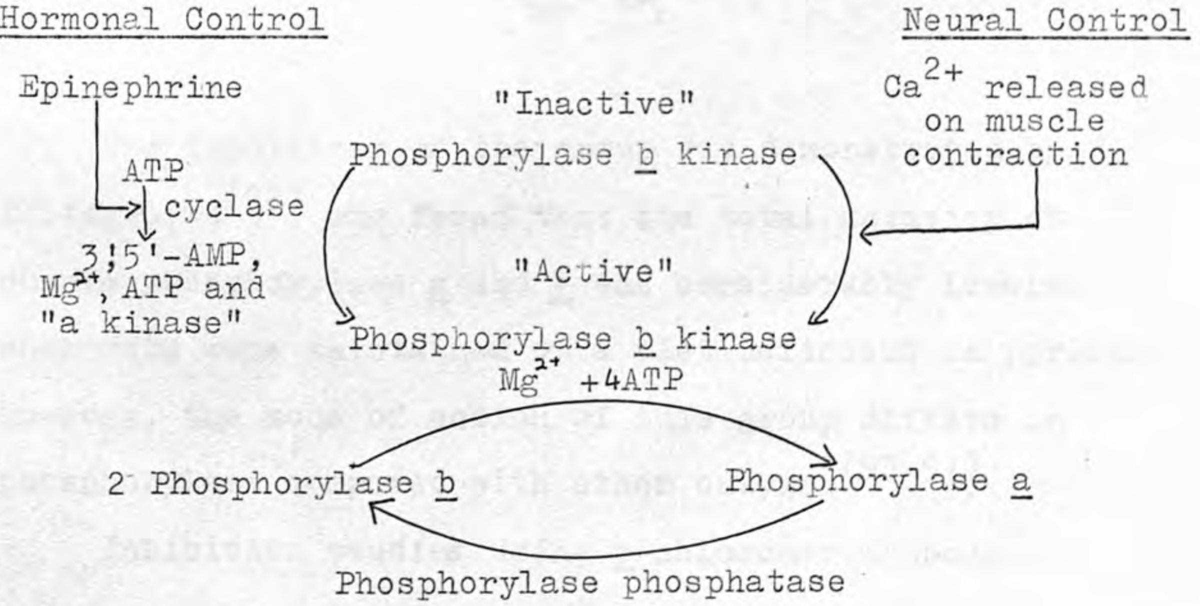
Krebs and Fischer[81] crystallised muscle phosphorylase b and converted it into phosphorylase a with a cell-free protein extract from muscle, together with bivalent ions and ATP. This enzyme, phosphorylase b kinase (ATP phosphorylase phosphotransferase) catalysed the irreversible

reaction:

2 Phosphorylase b + 4ATP → Phosphorylase a + 4ADP

During this reaction the molecular weight of the muscle phosphorylase doubles[82]. An important factor in the crystallisation of phosphorylase b is the dimerisation of 2 molecules of phosphorylase b each with two molecules of AMP[83] so that each molecule of phosphorylase a contains four molecules of AMP. Phosphorylase b kinase was later purified[84] and found to be activated by calcium ions[85] and cyclic 3',5'-adenosine monophosphate.

Cori[86] found that stimulated muscles converted phosphorylase a to b so that no phosphorylase a could be isolated from fatigued muscles, but resting muscle contained mostly phosphorylase b[87]. As a result of this, the following mechanism for the activation of glycogenolysis was suggested[88].



Whilst determining the structure of muscle phosphorylase, Baranowski and co-workers[89] found that each molecule of phosphorylase <u>a</u> contained eight molecules of inorganic phosphate. Four of these phosphates were extracted with trichloracetic acid and were shown to be present as pyridoxal-5-phosphate. Cori and Illingworth confirmed this[90] and showed that on removal of these groups the enzyme became inactive, but that reactivation could be effected by incubation with pyridoxal-5-phosphate. Kent and co-workers[91] suggested that the pyridoxal-5-phosphate was bound to the phosphorylase thus:

PROTEIN
HN X
CH
$H_2O_3P-O-CH_2$ OH
N CH_3

The importance of the group was demonstrated by Illingworth[92] who found that the total activity of muscle phosphorylase <u>a</u> and <u>b</u> was considerably lowered when rats were maintained on a diet deficient in pyridoxal. However, the mode of action of this group differs in phosphorylase compared with other enzymes[93.94].

Inhibition studies using <u>p</u>-chloromercuribenzoate

(p-CMB) indicated that phosphorylase a contained eighteen sulphydryl groups[95], half reacting rapidly, the rest more slowly. During the treatment with p-CMB the enzyme was split into four parts, each of molecular weight 125,000, a process which was reversed by the action of cysteine[96].

Elucidation of the amino acids of the active site of muscle phosphorylase gave the sequence[97]:

```
Ser-Asp-Glu-Glu-Lys-Arg-Lys-Glu-Ileu-Ser-Val-Arg-Gly-Leu
        |                   |
       NH2                 NH2
```

The isolation and purification of liver phosphorylase was finally achieved by Sutherland and Wosilait in 1956[98], but the enzyme was found to differ from muscle phosphorylase in having a lower molecular weight of 237,000[99]. It was, however, deactivated by the removal of approximately two phosphate ions per molecule of phosphorylase using a phosphatase, also isolated from the liver[100]; this latter reaction could not be reversed by AMP. Experiments using radioactive phosphate showed that phosphoserine was present in the liver phosphorylase, and that this serine phosphate did not exchange with inorganic phosphate during the catalytic reaction of the enzyme[101,102].

The dephosphorylated phosphorylase was reactivated by another enzyme, phosphokinase[102] which itself was

found to require a thermo-stable dialysable cofactor, identified as adenosine-3',5'-phosphate[103]. Administration of the cofactor to rats produced an increase in liver phosphorylase activity, with a simultaneous decrease in glycogen, and therefore it was suggested that it plays an important role in the regulation of glycogen metabolism[104].

Identification of the amino acids of the active site of liver phosphorylase showed a different sequence from muscle phosphorylase[105].

Muscle - Lys-Glu-Ileu-Ser-Val-Arg (with NH_2 on Glu)

Liver - Arg-Glu-Val-Ser-Val-Arg (with NH_2 on Glu)

This difference is also evident from immunological studies[106], and other experiments have shown that muscle phosphorylase prefers to degrade larger glycogen molecules than does the liver phosphorylase[107]. Calculation has shown that one phytoglycogen molecule of molecular weight 20×10^6 can bind up to thirty three molecules of muscle phosphorylase[72].

The action of phosphorylase is reversible although it is now believed that in the body its main function is the degradation of glycogen[108]. Muscle phosphorylase synthesises an unbranched chain of α-1,4-linked D-glucose

Figure II

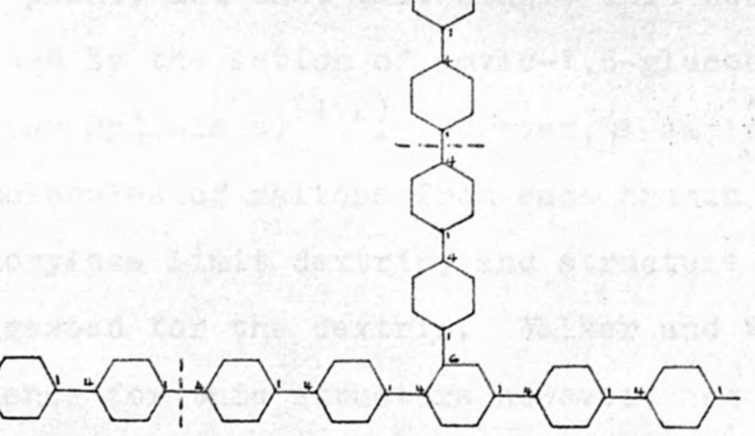
Phosphorylase Limit Dextrin

Cori and Larner Structure

Structure I

Walker and Whelan Structure

Structure II



--- — Linkages cleaved by β-amylase

units, but to produce a glycogen-like structure a second, branching enzyme must be present[109]. This was identified as amylo-1,4→1,6-transglucosidase[110] (α-1,4-glucan: α-1,4-glucan-6-glucosyltransferase), which was present as an impurity in the early crude phosphorylase preparations. Later investigation showed that if pure muscle phosphorylase was incubated with α-D-glucose-1-phosphate and adenylic acid a lag period occurred before any synthesis took place, but the addition of glycogen abolished this, and with the addition of amylo-1,4→1,6-transglucosidase a glycogen-like polysaccharide of molecular weight 2-25 x 10^6 was synthesised[111].

The degradative action of phosphorylase on glycogen was believed to be the removal of glucose units as α-D-glucose-1-phosphate from the A chains to within one unit of the branch point, and that this single unit could then be released by the action of amylo-1,6-glucosidase (starch 6-glucanohydrolase)[112]. However, β-amylase removed two molecules of maltose from each branch point of the phosphorylase limit dextrin, and structure I was therefore suggested for the dextrin. Walker and Whelan[113] found no evidence for this structure however, and suggested instead that the A and B chains were of equivalent lengths (Structure II) and that an amylo-1,4→1,4-glucantransferase had converted II into I by removal of a maltotriose unit. Such a glucantransferase has been

reported to occur as an impurity in amylo-1,6-glucosidase preparations[114]. Proof of the four unit A chain was obtained by observing the action of pullulanase, (which cleaves α-1,6-linked glucose units) on the dextrin, which produced maltotetraose as the main product[115].

Ample evidence of the degradative function of phosphorylase comes from glycogen storage diseases where there is a lack of phosphorylase and an accumulation of glycogen, thus indicating that glycogen synthesis probably proceeds by a different mechanism[116].

The debranching enzyme, amylo-1,6-glucosidase[117], was isolated by Cori and Larner[112] in 1951 although it had been present as an impurity in the phosphorylase preparations of Swanson[118]. The mode of action of this enzyme is the removal of the D-glucose unit in the A chain remaining after the action of phosphorylase and glucantransferase, so that the presence of all three enzymes is probably required to completely degrade glycogen <u>in vivo</u> to α-D-glucose-1-phosphate. However, recent investigations have suggested that the glucantransferase activity is a function of the amylo-1,6-glucosidase[119].

Although the action of amylo-1,6-glucosidase was originally believed to be irreversible, evidence of reversibility has recently been advanced by Hers[120]. Petrova[121] has also isolated an enzyme capable of

cleaving 1,6-links, from rabbit muscle extract, which was reversible.

The next stage in the catabolism of glycogen is the conversion of the glucose-1-phosphate, released by the phosphorylase, into glucose-6-phosphate. This is accomplished by the enzyme phosphoglucomutase (α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase) which is the only enzyme now believed to take part in both the synthetic and degradative pathways of glycogen metabolism.

Phosphoglucomutase activity was first noted in muscle extracts by Cori and Cori in 1936[122], and was subsequently detected in mammalian and frog tissues and in yeast[123]. In 1941, Sutherland and co-workers[124] showed that the enzyme action was reversible and later confirmed this by synthesising a "glucan" from glucose using hexokinase, phosphoglucomutase and phosphorylase[125].

While investigating yeast Caputto and co-workers[126] noted that the conversion of glucose-1-phosphate to glucose-6-phosphate only took place in the presence of a heat stable cofactor, α-D-glucose-1,6-diphosphate[127] which always occurred in small amounts together with the phosphoglucomutase[128]. Confirmation that glucose-1,6-diphosphate was behaving as a coenzyme was achieved using radioactive phosphate[129]. The purified enzyme was found

to contain non-dialysable phosphate which could be exchanged with ^{32}P-phosphate from glucose-1-phosphate[130].

On this evidence the following mechanism was postulated for the action of phosphoglucomutase[131]:

G-1-P + phosphorylated Enzyme → G-1,6-diP + dephosphorylated Enzyme

G-1,6-diP + dephosphorylated Enzyme → G-6-P + phosphorylated Enzyme

Phosphoglucomutase was crystallised by Najjar[132] in 1948 and its molecular weight shown to be 74,000[133]. Experiments showed phosphoglucomutase to be specific for glucose-1- and -6-phosphates[134], the velocity of the reaction increasing on addition of substrate up to a limit, after which it decreased, although the V_{max} depended on the concentration of the coenzyme[135].

An investigation of the structure of phosphoglucomutase using radioactive phosphate showed that the phosphate was firmly bound to the protein, and that while it was acid stable, it was labile in dilute alkali.[136] The sequence of amino acids of the active site of rabbit muscle phosphoglucomutase was constructed from the analysis of ^{32}P labelled phosphopeptides derived from the partial acid hydrolysis of the labelled enzyme[137].

Glu-(Asp,Leu)-Gly-Val-Thr-Ala-Ser-His-
--Asp-Gly-Glu-Ser-Ala-Gly-Leu-Asp-Leu-

From this structure it was evident that the catalytic site contained two functional serines on one peptide chain separated by four amino acid residues. Each serine was isolated as the phosphate ester and since each enzyme contained only one phosphate group it was suggested that the enzyme might exist in two forms depending on which of the two serines was phosphorylated[137].

Photo-oxidation of one particular methionine group in phosphoglucomutase reduced the activity of the enzyme below detectable limits, but oxidation of other methionines and histidines had no effect[138]. Comparative studies of phosphoglucomutases from different sources suggest that the structures vary slightly although the catalytic properties are similar[139].

The last enzyme concerned in the degradation of glycogen to glucose, glucose-6-phosphatase (α-D-glucose-6-phosphate phosphohydrolase), was only isolated in 1950[140], after de Duve and co-workers[141] had noted the presence of a phosphatase in the liver, which was specific for glucose-6-phosphate, with little or no action on glucose-1-phosphate, fructose-6-phosphate or β-glycerol phosphate. The mechanism of glucose-6-phosphatase action was

suggested to be as follows:[142]

$$\text{Enzyme} + \text{G-6-P} \rightarrow \text{Enzyme-G-6-P}$$
$$\text{Enzyme-G-6-P} \rightarrow \text{Enzyme-P} + \text{G}$$
$$\text{Enzyme-P} + H_2O \rightarrow \text{Enzyme} + P_i$$

Liver glucose-6-phosphatase was shown to be present in the microsomes[143,144], and on the basis of its specificity and location it was differentiated from non-specific acid and alkaline phosphatases[145]. Recent investigations have provided evidence that inorganic pyrophosphatase, glucose-6-phosphatase and pyrophosphate phosphotransferase have a common identity, as they could not be separated by ammonium sulphate fractionation[146,147], and their activities were similarly affected by hormone treatment[148,149]. Glucose-6-phosphatases having similar properties to the liver enzyme were found in the kidney and intestinal mucosa[150,145,151].

The enzyme concerned in the first step of the conversion of glucose to glycogen is a hexokinase,(ATP: D-hexose-6-phosphohydrolase) which in the presence of ATP converts some hexoses into the corresponding hexose-6-phosphates. This enzyme has been found widely distributed in the animal body; the enzyme in the brain, as well as yeast, phosphorylates D-mannose and D-fructose in addition to glucose[152]. In the liver however, a specific gluco-kinase was found[152] and Dipietro[153], using radioactive

compounds, measured the Michaelis constant of this enzyme and showed that it was probably responsible for most of the glucose phosphorylation in rat liver[154].

The presence of both a hexokinase and a glucokinase in the liver has been confirmed[155], and investigation of the cellular locations in the liver showed a distinct separation[156]. The glucokinase was purified[157], and found to be affected by nutritional factors[158], being depressed by scurvy and diabetes[159,160], but the activity was returned to normal on treatment with insulin. During these processes the activity of the liver hexokinase remained virtually unchanged[160]. In contrast, muscle tissues contain only a hexokinase which is inhibited by glucose-6-phosphate, but is not affected by insulin[156]. Comparative studies on mammals have shown that the occurrence of the specific glucokinase in the liver is dependent on the species[161].

Until 1957 it was believed that glycogen was both synthesised and degraded by phosphorylase. Then Leloir and Cardini[75] identified an enzyme in liver which, when incubated with a primer molecule and uridine diphosphate glucose produced glycogen[162].

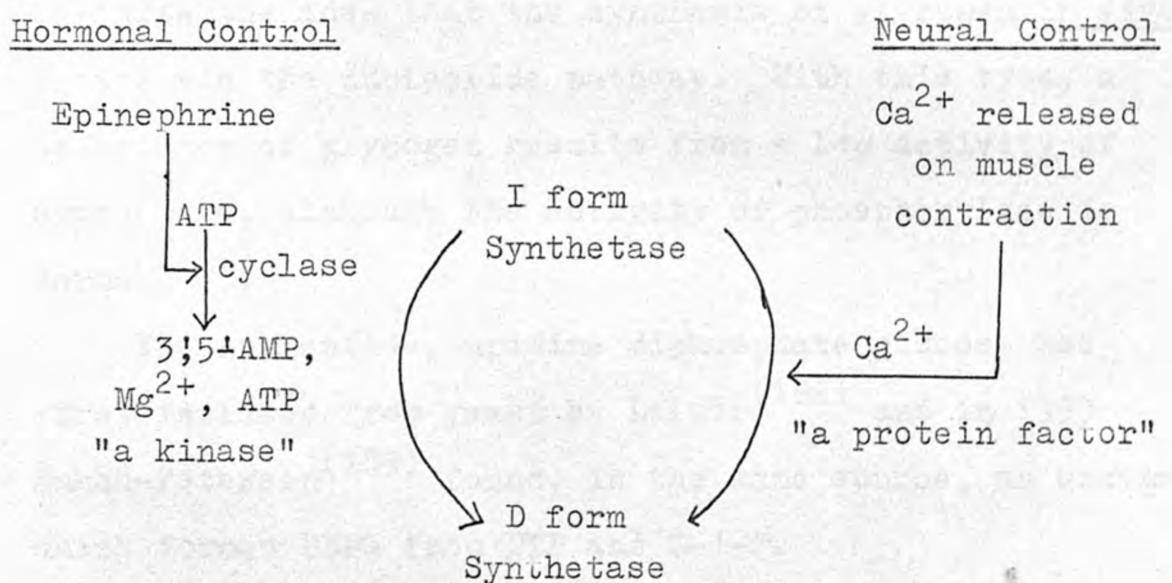
$$UTP + G\text{-}1\text{-}P \rightarrow UDPG + P\text{-}P$$

$$UDPG + G_n \rightarrow UDP + G_{n+1}$$

The enzyme, glycogen synthetase (UDPG:α-1,4-glucan α-4-glucosyltransferase), was also found in muscle tissue[163], mammary gland[164], uterus[165], brain[166] and in pigeon breast muscle[167], although muscle tissues contained less synthetase than phosphorylase[163]. The activity of the synthetase was mainly concentrated in the microsomal particles[167], but on centrifuging, the enzyme was found to sediment together with the glycogen[168].

The action of the enzyme is to add single D-glucose units on to a glycogen primer with α-1,4-linkages[169], by a multichain mechanism[170], the process being enhanced by Mg^{2+} ions and glucose-6-phosphate[171]. At first the significance of this latter enhancement was not fully realised until Friedman and Larner[172] separated the enzyme from the liver into two forms, one being active without glucose-6-phosphate (I form), the other depending on glucose-6-phosphate for activity (D form). A third form, inactive even in the presence of glucose-6-phosphate, has also been isolated[173]. These first two forms, I and D, can be interconverted by dephosphorylation of I to give D, and reconverted by ATP in the presence of Mg^{2+} ions[172]. Using purified enzyme[174], the specificity of the activating sugar phosphate was investigated[175]; a monosaccharide in the pyranoid form with free hydroxyl groups at C-2, C-3, and C-4, and a phosphate group on C-6 was required in order to produce activation.

An enzyme associated with the particulate glycogen was found which, in the presence of Mg^{2+} ions, converted the D to the I form[173]. This enzyme, when highly purified needed another factor for activity[173] which was very similar to that involved in the activation of inactive (dephosphorylated) phosphorylase b kinase by calcium ions[176]. However, purification of both synthetase and phosphorylase b kinase from muscle[177], and experiments using insulin have shown that the enzymes are not identical. Therefore the mechanism for the activation and deactivation of glycogen synthetase is postulated to be as follows:[178]



The primer molecule specificity of synthetase varies depending on the source of the enzyme; yeast synthetase shows a lack of activity with oligosaccharides[179], although rat muscle synthetase can act on oligosaccharides as small as maltotetraose, synthesising maltopentaose, indicating a multichain mechanism[180].

The amino acid sequence of the phosphorylation site of synthetase appears to be

$$\left.\begin{matrix}\text{Arg}\\\text{Lys}\end{matrix}\right\}\text{-Glu-Ileu-Ser-Val-Arg}$$

being similar in both rat and rabbit muscle tissue[181].

One particular type of Glycogen Storage disease supports the idea that the synthesis of glycogen <u>in vivo</u> occurs via the nucleotide pathway. With this type, a deficiency of glycogen results from a low activity of synthetase, although the activity of phosphorylase is normal[108].

The nucleotide, uridine diphosphate glucose was first isolated from yeast by Leloir[182] and in 1953 Munch-Petersen[183] found, in the same source, an enzyme which formed UDPG from UTP and G-1-P.

$$\text{G-1-P} + \text{UTP} \rightarrow \text{UDPG} + \text{P-P}$$

This enzyme, UDPG pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase) was purified from yeast[184]

and the reaction of the enzyme was confirmed using ^{32}P labelled phosphate. On investigating animal tissues, similar enzymes were detected in the skeletal and diaphragm muscles[162], liver[185] and brain[186,187].

The presence of a branching enzyme synthesising the α-1,6-links in glycogen was first suggested by Cori, who found that whereas muscle phosphorylase synthesised straight chain amylose, liver and heart phosphorylase preparations synthesised glycogen, due to an impurity in these extracts. Similar enzymes have been detected in adipose tissue[188], and other tissues[189].

The mode of action of this enzyme (amylo-1,4-1,6-transglucosylase, α-1,4-glucan:α-1,4-glucan-6-glucosyl transferase) was investigated using glycogen labelled with radioactive glucose units in the outer chains[110]. On incubation it was observed that the branching enzyme transferred not a single glucose unit but chains, the smallest chain being six glucose units long[190]. For transfer to occur however, the outer chains were required to consist of at least 11-12 glucose units, and the addition of maltose, maltotriose or panose to the reaction mixture produced no stimulation indicating that the enzyme had a specific substrate requirement[191].

Glycogen has also been reported to be synthesised

and degraded by other enzymes. Petrova detected an enzyme in rabbit muscle which synthesised a glycogen-type carbohydrate from glucose and dextrins[192], while another was found in rat liver[193] which synthesised oligo-saccharides from maltose with the continuous liberation of glucose.

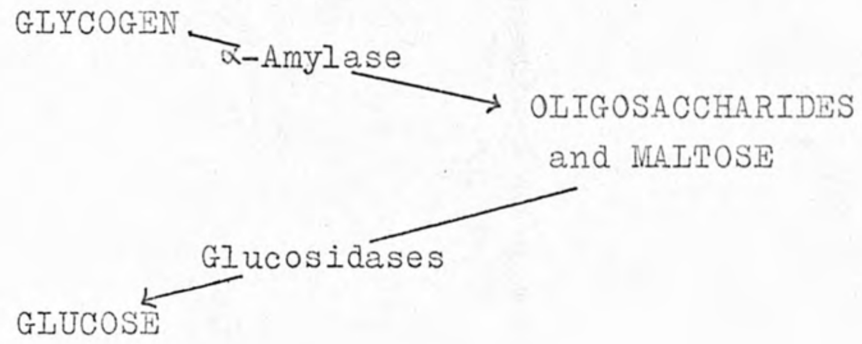
$$G\text{-}G + G\text{-}G \rightarrow G\text{-}G\text{-}G + G$$

Enzymes capable of degrading glycogen have been found in muscles[194] and liver[195]. Olavarria[196], in 1960, noted that after the addition of labelled UDPG to a liver extract, the radioactivity was found in both glycogen and oligosaccharides, suggesting that the oligo-saccharides had been formed as breakdown products of the glycogen, by the action of an α-amylase-type enzyme. An amylase was identified from a liver extract which hydrolysed glycogen, first to produce the linear oligo-saccharides maltotriose, -tetraose and -pentaose and some maltose from the outer chains of glycogen; then branched oligosaccharides by the hydrolysis of the core of the glycogen molecule[197].

The same workers also isolated two glucosidases[198] from dog liver, one, an acid glucosidase, which hydrolysed glycogen and glucosides, and a neutral glucosidase, which also hydrolysed these compounds, but cleaved maltose

preferentially to other glucose disaccharides.

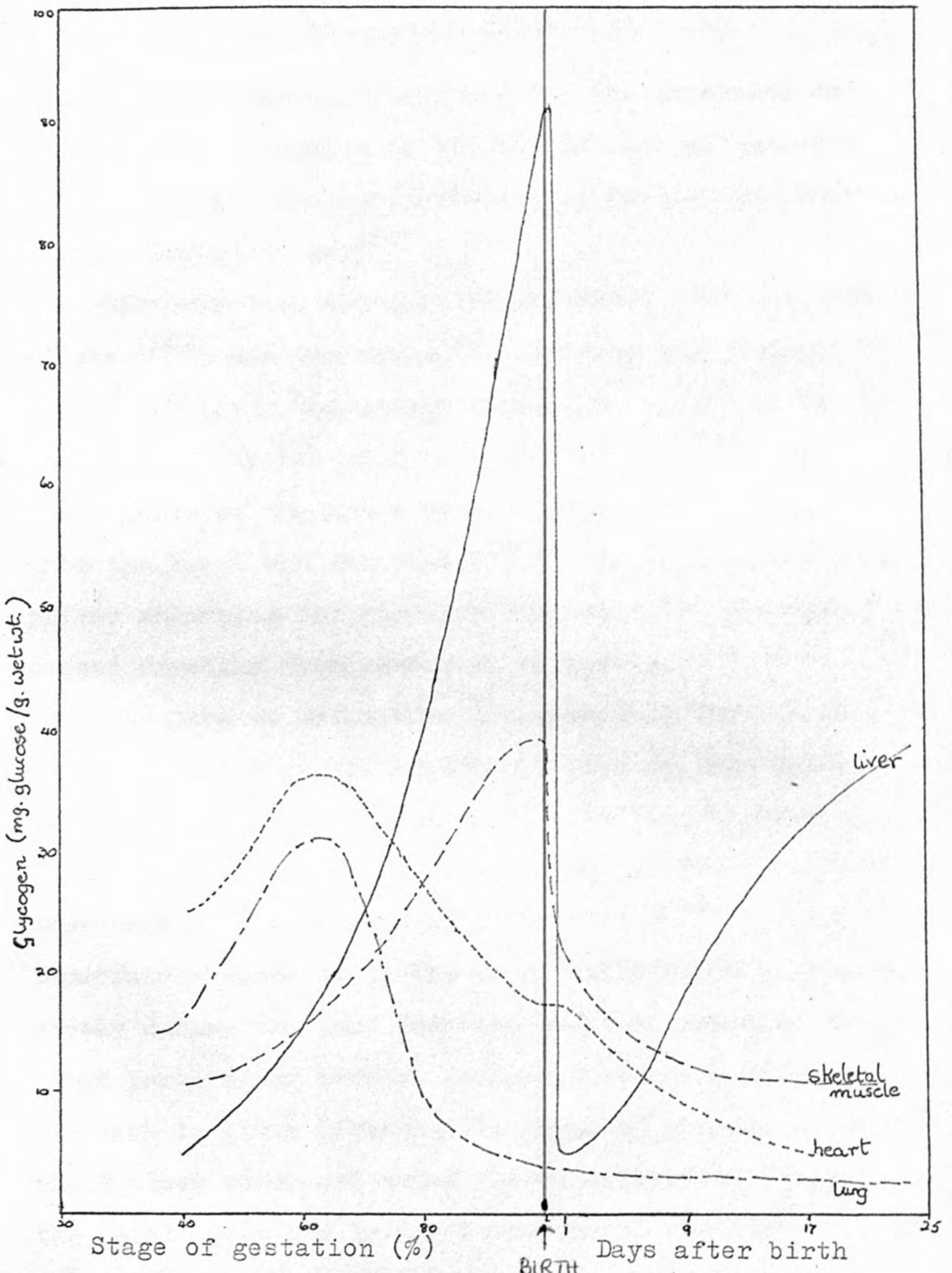
A mechanism for the degradation of glycogen not involving phosphorylase was suggested to be:



The presence of these two glucosidases, also called maltases was observed in human liver tissue(199), and the lack of the acid glucosidase in the lysosomal cells(198) has been shown to be responsible for Type II (Generalised) Glycogen Storage Disease(200).

Figure III

Glycogen in the Body Tissues of a Sheep



DEVELOPMENT OF GLYCOGEN METABOLISM

The development of enzymes for the synthesis and metabolism of glycogen in the tissues is particularly interesting and has been extensively studied in numerous species including man[201].

Histochemical examination has shown that both the oöcyte [202] and the sperm[203] contain the glycogen needed for their own energy requirements, and it is also present in the nucleus of the oöcyte[204] and in the tissues of the human embryo which later develop into the heart and intestines[205]. Hence the necessary primer molecules for glycogen synthesis are presumably passed directly from parent to offspring.

The time of appearance of measurable amounts of glycogen during gestation depends both on the tissue concerned and on the species. In the liver little glycogen is found early in gestation but the amount increases in the later stages until term[201]. Immediately after birth the level falls rapidly, rising slowly during the next few days and only reaching the adult level after several weeks. Concurrent with the decrease in liver glycogen the level of glucose in the blood rises to almost twice the concentration found in the fetal blood due to the breakdown of the liver glycogen.[205]

A similar pattern of increasing glycogen storage during gestation has been observed in skeletal muscle but unlike the liver the level of glycogen does not rise again after birth[205]. A different metabolic pattern occurs in the heart and lungs. In these tissues the glycogen level rises during the first half of gestation, but then falls during the latter half to a low value[206,207]. In fetal brain, kidney and skin the glycogen level is higher than in the adult, but no complete metabolic studies have yet been made of these organs[204,206,208].

Investigation has shown that the regulation of the level of blood sugar in the fetus is controlled by the placenta during the first part of gestation when it contains a relatively high concentration of glycogen[209,210], but this level in the placenta falls at about the same time as glycogen is first detected in the fetal liver. This suggests that at that time the fetus has developed sufficiently so that it can metabolise glycogen, thereby controlling its own glucose level[211].

In the human fetus, glycogen has been detected histochemically in the liver as early as six weeks by Shapovolov[212], although Kitamura[213] did not find any until four months. Bourne, McLean and Pridham[214] were able to extract glycogen from human fetal livers aged 13½ weeks; the amount of trichloracetic acid extractable glycogen increased up until term.

Analysis of fetal goat[215] and human glycogens[214] showed that the structure was similar, if not identical, with that of the adult, but the molecular weight of embryo calf muscle and liver glycogen was found to be less than one half that of the adult polysaccharide[216].

The levels of activity of the enzymes concerned in fetal metabolism have also been followed in an attempt to explain the increase in storage of glycogen during gestation. Due to the diminutive size and the scarcity of human fetal tissues, the tissues of small mammals have been investigated more thoroughly.

Although hexokinase is present in fetal liver during gestation[217,218], glucokinase is only found after birth[219,220]. Phosphoglucomutase, on the other hand, and its coenzyme, is present before birth[217], with a lower activity than in the adult[221], while the levels of glycogen synthetase and UDPG pyrophosphorylase, although much lower in the fetus than in the adult[222], increase rapidly just before term and remain high after birth[223]. In the liver, heart and skeletal muscle of the chick embryo the synthetase activity is detected at the same time as the appearance of glycogen, but phosphorylase is found to develope later[224,225]. The opposite occurs in fetal rat liver where phosphorylase is detected before the deposition of glycogen, the activity rising slowly, in

parallel with phosphorylase stimulating substances[226] until term, and then reaching the adult level within twenty-four hours after birth[227]. Guinea-pig phosphorylase activity follows another pattern, increasing slowly during gestation then rapidly at birth up to a level on the 2nd postnatal day, of three times that of the adult, and finally falling to the adult level by the 6th day[222].

In sheep the synthetic enzymes, synthetase and UDPG pyrophosphorylase, were found to have higher activities in the fetus than in the adult, while the activities of phosphoglucomutase and phosphorylase were lower in the fetus than in the adult[228].

Glucose-6-phosphatase, the last enzyme concerned in the degradation of glycogen to glucose, exhibits very little activity, or appears to be completely absent from the livers of humans[229], rats[230] and guinea-pigs[231] during gestation. At birth the activity suddenly increases to a level equal or greater than that of the adult animal[227], and only falls to the "normal" level after several weeks[232,233]. Similar rises in activity at birth have also been observed in the kidney[231].

This increase in activity of glucose-6-phosphatase coincides with the sudden decrease of glycogen in the liver, and these two processes are believed to be

related to birth itself, as the glycogen level of prematurely born humans does not fall after birth[213], and in some still-born infants the glycogen is not metabolised by the liver itself, although it is degraded by the liver pulp of adult guinea-pigs[234]. This suggested that the ability to produce glucose-6-phosphatase occurs only when the fetus is carried for the full gestational period and survives after birth.

Investigation of the hormonal control of glycogen storage in the fetal liver has shown that adrenalectomised fetuses failed to store any glycogen, but adrenalectomy of the dam after the commencement of glycogen storage in the fetal liver has no effect on the fetal deposition of glycogen, thus indicating that when the fetus has the ability to deposit glycogen in the liver, it is also able to control its own glycogen metabolism[235,236].

Glycogen storage in fetal mouse liver can however be lowered by starvation of the dam but the decrease is less in the fetal liver than in that of the dam[237].

Glycogen metabolism in an adult animal can be affected by such factors as nutrition, the administration of drugs and pathological conditions. During starvation the activities of glycogen synthetase[238] and glucokinase[239] decrease in the liver while the activity of glucose-6-phosphatase increases[240] causing a lowering of the

glycogen level in the liver. The largest decrease of glycogen has been shown to occur in the liver with smaller decreases in the muscles, heart and spleen; the other tissues were relatively unaffected[241]. A slight decrease in the activity of phosphorylase has also been observed during periods of starvation[242,243].

The activity of glucose-6-phosphatase also increases and the glucokinase decreases[244] if the diet contains protein, fat, galactose or fructose instead of a direct glucose source[245]. If a high glucose diet is administered then a rise in the level of glucokinase is observed[244], while on a phosphate deficient diet the activity of glucose-6-phosphatase increases[246].

It was observed[247] that as the level of muscle glycogen decreased so the conversion of the D→I form of glycogen synthetase was promoted, suggesting that, due to the tight binding of synthetase to glycogen, a decrease in the glycogen made the synthetase more accessible to the conversion enzymes.

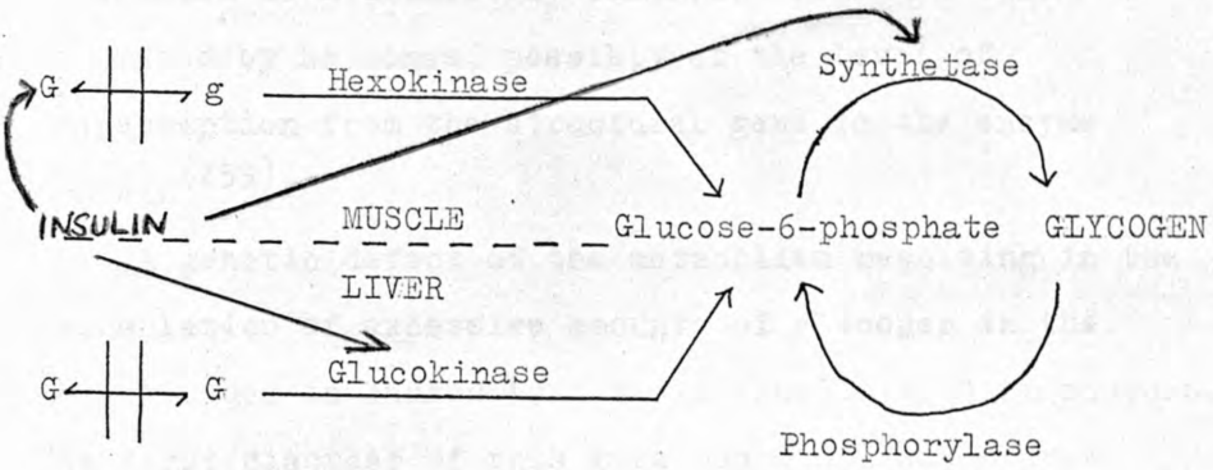
PATHOLOGICAL CONDITIONS AFFECTING GLYCOGEN METABOLISM

The most common disease affecting glycogen metabolism is diabetes mellitus, which is manifest as an impairment in the glucose utilisation by the liver, resulting in a decrease in the synthesis of glycogen and fatty acids[248]. This is due to a deficiency of insulin, and attempts have been made to elucidate the specific action of insulin.

In the diabetic liver the activity of glucose-6-phosphatase is elevated[249] but glucokinase is depressed[153] and on administration of insulin the activities return to normal[249,160]. These changes in the activities result in a reduced deposition of glycogen and an excessive concentration of glucose in the blood. Insulin also promotes the activity of synthetase in muscles[250] and liver[251] but has no effect on phosphorylase and phosphoglucomutase in diaphragm muscles[252]. In skeletal muscle, insulin stimulates glucose uptake by accelerating glucose transport through the cell membrane[253], but as the liver is freely permeable to both the inflow and outflow of glucose[254], two different mechanisms have been suggested for the regulation of the glycogen metabolism by insulin[156].

REGULATION OF GLYCOGEN METABOLISM BY INSULIN

→ = promoting action of insulin



The effect of other hormones on glycogen metabolism has also been extensively investigated, and has resulted in the grouping of hormones into two types depending on their action. Epinephrine and glucagon, examples of one type, promote glycogenolysis by stimulating the activation of phosphorylase[255,103]; the former hormone also reduces the activities of both the I and D forms of synthetase[252]. The other type of hormone such as cortisone and insulin, promotes glycogen deposition[256].

Other effects which have been observed are the promotion of glucose-6-phosphatase activity by o-tyrosine[257],

and a decrease in the level of liver and kidney glycogen on treatment with phloridzin[258].

It has therefore been suggested that glycogen metabolism is a delicately balanced mechanism which is regulated by hormones, possibly at the level of transcription from the structural gene to the enzyme protein[259].

A genetic defect of the metabolism resulting in the accumulation of excessive amounts of glycogen in the body tissues is caused by a deficiency of certain enzymes. The first disorder of this type was described by van Creveld in 1928[260]. The following year von Gierke[261] characterised a condition of enlargement of the liver and kidney due to excessive storage of glycogen, and so the disease became known generally as glycogen storage, or von Gierke's disease. Subsequently, numerous other cases have been reported, and as assay techniques developed, so it became possibly to classify the types of disease on an aetiological basis.

Type I

This, the most common form of glycogen storage disease, was named after von Gierke who described the first case[261]. Schoenheimer, investigating the tissues of von Gierke's patient, showed that the storage was due to an enzyme deficiency[262]. The general features of

the disease are an accumulation of glycogen up to 16% in the liver and kidneys due to a deficiency of glucose-6-phosphatase. The glycogen, however, has a normal structure.[265]

Other cases lacking glucose-6-phosphatase have been described[266,267], some showing a degradation of glycogen to lactate instead of glucose[268,269], thereby by-passing the glucose-6-phosphatase step. Cori[263] was unable to explain the absence of enzyme activity by a deficiency of cofactors or the presence of an inhibitor. However, as the enzyme defect appears concurrently in the liver and the kidney, it has been suggested that it is controlled by the same gene[270], and that the disease is inherited as a recessive gene trait[271].

Type II

Pompe's disease is characterised by generalised glycogen storage affecting the cardiac and skeletal muscles, and the liver[272]; the disease being fatal in the first or second year of life due to the involvement of the heart muscle. The structure of the glycogen was found to be normal and the accumulation of glycogen was shown to be due to an absence of an acid α-1,4-glucosidase[199], which is present in human liver, heart and muscle tissues[275], and in rat liver is located in the lysosomes[168]. In the absence of this enzyme glycogen accumulates in vacuoles, as found by Hers[276].

Type III

Illingworth and Cori[264] conducted the first chemical investigation of this type of disease and found that the glycogen had a "limit dextrin" structure with an $\overline{ECL}$ of 5.1 glucose units and an $\overline{ICL}$ of 4.2 units. Later investigations[274] showed that this was due to a specific deficiency of the debranching enzyme amylo-1,6-glucosidase, which could occur in either the muscles[263] or the liver[277] or both[278,279]. Further structural analysis of these "limit dextrins" indicated that they were of two types, one with a Cori-Larner type structure with short outer chains of less than four glucose units[280], the other having the Walker-Whelan structure with longer outer chains of 4-5 glucose units[263,281], see Page 29.

This suggested that two types of limit dextrinosis existed, one due to a lack of amylo-1,6-glucosidase, and another due to a lack of the transglucosidase and amylo-1,6-glucosidase giving limit dextrins of different chain length[281].

Re-examination in 1963 of the original two cases of van Creveld showed that they belonged to Type III rather than Type I and although the patients were then adult some abnormality in their carbohydrate metabolism was still evident[282]

Type IV

Only a very few cases of this type have been reported[283,284]; it is due to a deficiency or defect in the branching enzyme amylo-1,4→1,6-transglucosidase in the liver and other tissues. This results in the synthesis of a "glycogen" with an amylopectin type structure having an average outer chain length of 14,7 glucose units. As this polysaccharide would be less soluble than glycogen in water, it was suggested that it precipitated out, becoming inaccessible to degradation by phosphorylase, and therefore accumulated[279].

Type V

McArdle's disease is confined entirely to the skeletal muscle and was diagnosed as being due to a lack of phosphorylase[285], although the activities of phosphorylase kinase and phosphatase were present[286]. The activities of UDPG pyrophosphorylase, amylo-1,6-glucosidase and phosphoglucomutase are unaffected but in one particular case decreased activities of synthetase and phosphoglucomutase were noted.

The general manifestation of the disease is a lack of "energy"[287] and it can appear in adults as well as in children[288]. Examination of one family has shown that this deficiency is genetically determined and is due to an autosomal recessive gene[289]

Type VI

This disease is related to Type V in that it is also caused by a lack of phosphorylase, but in this case the glycogen accumulates in the liver[290,291]. Thus the phosphorylases of the muscle and liver are apparently under different genetic control.

Several other cases of glycogen storage disease have been reported due to enzymic lesions of amylo-1,6-glucosidase and phosphorylase in the leukocytes[292,293], and phosphofructokinase in the muscles[294]. Multiple defects have also been detected where there is a deficiency of both phosphorylase and glucose-6-phosphatase[295].

An interesting case concerning not glycogen storage but glycogen deficiency due to the lack of synthetase[108] has been reported. The activities of phosphorylase, UDPG-pyrophosphorylase and glucose-6-phosphatase were found to be normal but the concentration of liver glycogen was very low causing severe hypoglycaemia in the patient.

Two untyped storage diseases have also been reported in which the glycogen, of apparently normal structure accumulates in the muscle, but there is no deficiency of phosphorylase[296,297].

The mode of inheritance of glycogen storage disease due to a single recessive gene has not been fully elucidated as it appears that different genes control

the mechanism of the same enzyme in different tissues as in Type V and VI; in Type I the same deficiency occurs in both the liver and the kidney[270]. In addition, different types of storage disease have been found to occur in the same family[265,282].

Chieffi and Nassi[297] suggested that the lack of enzymes in the various tissues was due to a malfunction of the endocrine glands resulting in insufficient amounts of growth promoting hormone. Other workers have reported an alleviation of the symptoms by the daily injection of adrenocorticotropic hormone[298]. Injection of amylo-glucosidase into rats has been shown to produce a decrease in the glycogen content of the muscle, heart and liver[299], but as yet no "cure" for any of the diseases is known.

MAIN SECTION

(i) Studies on Glycogen

Since Bernard first isolated glycogen in 1857[9], investigations have been made to determine the structure of the glycogen molecule, and to elucidate the pathways by which glycogen is synthesised and degraded in the liver and other organs. Considerable research has also been carried out on the physiological state of glycogen in the tissues as the existence of two forms of glycogen had been suggested, one of which was easily extractable, and a less readily extractable form which was thought to be "bound" to protein[70]. More recently however, interest has centred on the development in the fetus of the enzymic pathways necessary for the synthesis and metabolism of glycogen.

The purpose of this research on rat and human liver tissue was (i) to investigate the development of certain enzymes during fetal life, (ii) to determine the proportions of extractable and residual glycogen in the fetal liver, and (iii) to examine the structure of this extractable and residual glycogen.

The partial structure of glycogen was elucidated by early workers using procedures such as methylation and periodate oxidation. More recently these techniques have been replaced by enzymic methods, thus enabling

milligram quantities of glycogen to be analysed.

In order to gain experience with these methods a number of glycogens were analysed and it was shown that the average chain lengths ($\overline{CL}$) of the polysaccharides varied between 10.1 → 14.0 D-glucose units, and the average internal ($\overline{ICL}$) and external ($\overline{ECL}$) chain lengths between 2.1 →4.7 and 7.0 →8.9 D-glucose units, as shown below in Table I.

TABLE I

Structures of Glycogens

Sample	$\overline{CL}$	$\overline{ECL}$	$\overline{ICL}$	$\lambda_{max} m\mu$
Average[2]	10-14	7-9	2-6	420-490
Rabbit liver I	12.6	8.2	3.4	480
Rabbit liver II	12.8	8.1	3.7	480
Rabbit liver III	13.0	8.6	3.4	455
β-A.L.D.of II*	6.9	2.5	3.4	430
Child liver	14.0	8.3	4.7	450
Human fetal, 26 wk.	10.1	7.0	2.1	-
Glycogen Storage Disease				
Liver - G. R.	13.8	8.9	3.9	450
Liver - P. W.	13.0	7.5	4.5	460

*β-Amylase limit dextrin of rabbit liver glycogen II

All these values are within the "normal" range quoted by Manners[2], and are typical illustrations of the highly branched structure of glycogen.

Additional evidence of this was obtained by the stepwise degradation of glycogen with β-amylase and pullulanase. β-Amylase hydrolyses α-1,4-linked D-glucose chains to maltose[30], while pullulanase hydrolyses α-1,6-linked D-glucose units[300], its normal substrate being pullulan, a glucan containing both α-1,4 and α-1,6-linkages[301].

Pullulan

⬡ - α-D-glucose units

After three incubations alternately with pullulanase and β-amylase, almost all the original glycogen had been reduced to maltose and glucose (as shown in Table II), confirming the evidence of Larner et al.[33]. The amount of hydrolysis by the first incubation with pullulanase increased the percentage β-amylolysis from 43.5 to 47.8, a small amount[302], suggesting that pullulanase prefers to

TABLE II

Hydrolysis of Rabbit Liver Glycogen II

Incubations	% Hydrolysis - reducing sugars measured as maltose
β-Amylase alone	43.5
Pullulanase followed by β-amylase → Solution I	47.8
Pullulanase followed by β-amylase on Solution I → Solution II	61.8
Pullulanase followed by β-amylase on Solution II → Solution III	92.0

remove small glucan chains (cf action on pullulan[301]), rather than the longer chains which would be present on the exterior of the molecule[303]. If the longer chains were attacked, then one would expect the whole molecule to be degraded.

The action of pullulanase on β-amylase and glucamylase limit dextrins of glycogen is summarised in Table III.

TABLE III

Products of Hydrolysis

Limit Dextrin	Glucose	Maltose	Maltotriose	Maltotetraose
Glucamylase	++	++	+	trace
β-Amylase	-	++	++	+

The fact that maltose and maltotriose are the main oligosaccharides released from β-amylase limit dextrin provides confirmation that the average number of glucose units left as "stubs" after the action of β-amylase is 2.5 units[304]. This value is only average and therefore the production of a little maltotetraose was not unexpected. The liberation of glucose from the glucamylase limit dextrin shows that glucamylase is capable of greater degradation of glycogen than β-amylase; McLean[305] reported an average "stub" length of 1.5 glucose units. Again, as this is an average value the small amounts of maltotriose and -tetraose were to be expected.

Examination of the infrared spectra of several glycogens (Table IV) showed that all had the normal pattern of absorption in the region 750 - 1,000 cm^{-1}, with peaks at 759±4 and 926±4 cm^{-1} characteristic of 1,4-linked glucose units, and at 848±5 cm^{-1} characteristic of the α-D-glucosyl linkage[42]. There was no trace of absorption at 890 cm^{-1}, which is characteristic of the β-linkages.

An aqueous solution of iodine and potassium iodide has characteristic absorption peaks at 288 and 350 mμ, which are believed to be due to the presence of the $I_3^{\ominus}$ ion

TABLE IV

The Infrared Absorption Peaks of the Glycogens

Glycogen	Wavelength in cm^{-1}		
Human J.H.	758	848	926
Human J.B.	762	850	928
Human 26 wk. fetus	760	845	925
Human 1 wk. old	758	845	925
Human Storage Disease G.R.	760	845	928
Human Storage Disease L.	756	843	922
Mouse liver	760	850	925
Baboon liver	760	855	925
Calf liver	760	850	925
Rabbit liver I	765	845	930
Rabbit liver II	760	845	928
Rabbit liver III	758	848	926
β-Amylase limit dextrin of Rabbit liver II	758	850	925

produced in the equilibrium reaction[306],

$$I^{\ominus} + I_2 \rightarrow I_3^{\ominus}$$

and these absorption bands are very sensitive to concentration changes, and to the nature of the solvent.

The addition of glycogen to a solution containing this equilibrium mixture results in the formation of a glycogen-iodine complex, with a characteristic absorption between 420-490 mμ, for which a helical glucan chain is believed to be necessary[54]. However, addition of small amounts of methyl, ethyl or n-propyl alcohol to the glycogen-iodine complex resulted in a decrease in absorbance on a molar basis, the effectiveness being in the order ethanol$>$methanol$>$n-propanol. (Table V)

TABLE V

Decrease in the Glycogen-Iodine Complex Absorption

Alcohol	Molar decrease in Absorption
Methanol	3.4
Ethanol	8.6
n-Propanol	1.4

The addition of these alcohols to an aqueous solution of iodine in potassium iodide did not, however, affect the absorption due to the $I_3^{\ominus}$ ion, nor was this absorption affected when the iodine and potassium iodide were dissolved in ethanol. (Figure IV)

Treatment of a sample of glycogen, having an iodine complex absorption maximum at 460 mμ, with the Sevag reagent, used for the removal of protein[307], resulted in the loss of this absorption, which was presumably due to the adsorption of iso-amyl alcohol by the glycogen molecule. Removal of this iso-amyl alcohol from the polysaccharide by extraction with ether, and isolation of the glycogen, resulted in the reappearance of the absorption maximum at 460 mμ. Similar occlusions have been noted by Ullman and Schierbaum using starch[308].

A similar decrease in absorption of the glycogen-iodine complex was produced by the addition of urea, the decrease in the absorption being proportional to the amount of urea added and to the glycogen concentration. (Figure V) Urea itself, like the alcohols, had no effect on the absorption bands at 288 and 350 mμ (Figure IV), other than dilution effects, which resulted in slight shifts in the λ_{max}. The urea had only a small effect on the pH of the solutions.

Fig. IV. Identical Spectra of the Potassium iodide-Iodine Equilibrium in Alcohol. Water, and with 8M Urea

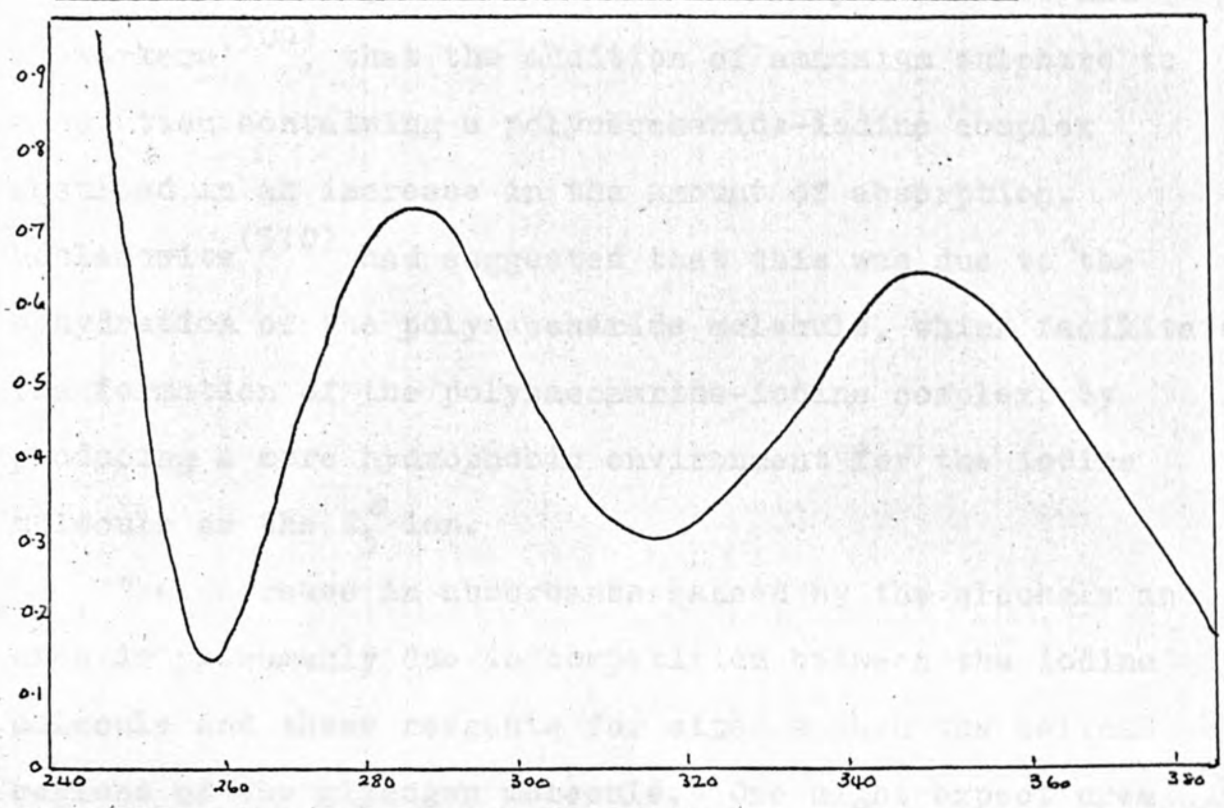
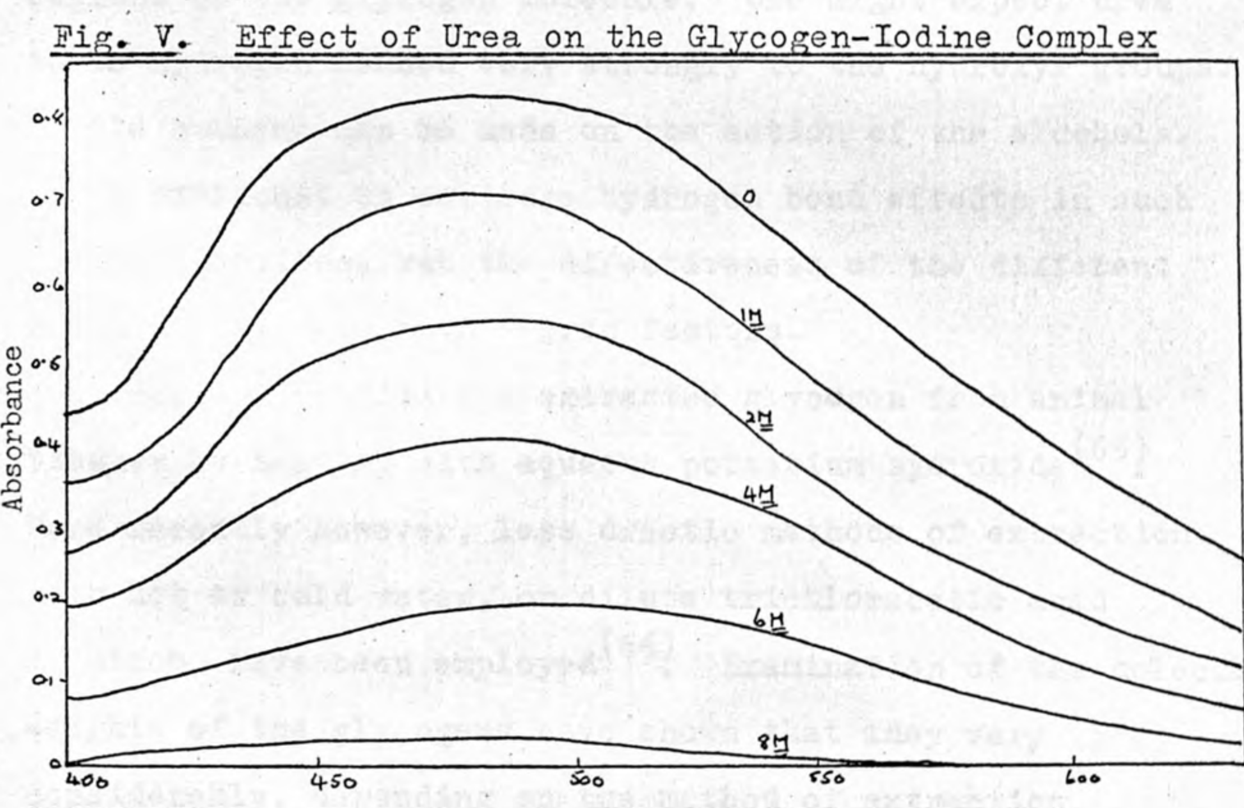


Fig. V. Effect of Urea on the Glycogen-Iodine Complex



A contrasting effect was observed by Archibald and co-workers[309], that the addition of ammonium sulphate to a solution containing a polysaccharide-iodine complex resulted in an increase in the amount of absorption. Schlamowitz[310] had suggested that this was due to the dehydration of the polysaccharide molecule, which facilitated the formation of the polysaccharide-iodine complex, by producing a more hydrophobic environment for the iodine molecule or the $I_3^{\ominus}$ ion.

The decrease in absorbance caused by the alcohols and urea is presumably due to competition between the iodine molecule and these reagents for sites within the helical regions of the glycogen molecule. One might expect urea to be hydrogen bonded very strongly to the hydroxyl groups. Little comment can be made on the action of the alcohols. It is difficult to envisage hydrogen bond effects in such dilute solutions, but the effectiveness of the different alcohols may depend on steric factors.

Early investigators extracted glycogen from animal tissues by heating with aqueous potassium hydroxide[65]. More recently however, less drastic methods of extraction with hot or cold water, or dilute trichloracetic acid solution, have been employed[66]. Examination of the molecular weights of the glycogens have shown that they vary considerably, depending on the method of extraction.

Extraction with cold water for example, yielded a glycogen with a high molecular weight of 450×10^6 [64], but if hot alkali or acid were used the molecular weight was considerably reduced, the reduction being greater with alkali than with acid[62,311].

During these experiments it was also noted that less glycogen was extracted from the tissues by water or dilute acid, than by hot alkali, and it was therefore suggested that the tissues contained two forms of glycogen, one being "free", the so-called extractable or lyoglycogen, while the other, termed residual or desmoglycogen, was thought to be bound to protein[29].

Considerable discussion has taken place over this question of the binding of protein and glycogen, and whether the two forms are metabolically dissimilar. Bloom and co-workers[312] investigated the proportions of extractable and residual glycogen in rat liver under different physiological conditions and found that the relative amounts of glycogen were consistent, provided the animals were kept under uniform conditions. However, if the nutritional state of the animal was altered by fasting, the extractable glycogen was more readily depleted during the fast, and was more readily restored on refeeding, than residual glycogen.

Further investigations were carried out to determine the effect of other physiological and pathological conditions on the proportions of extractable and residual glycogen in the tissues[73], and it was generally observed that the extractable glycogen was more affected than the residual glycogen, when the conditions were changed.

Experiments on the reactions of extractable and residual glycogen, conducted by Figueroa and co-workers[71], showed that 70% of the residual glycogen was precipitated from aqueous solution at 70°C. Extractable glycogen however, was not precipitated on heating to 100°, but both forms were precipitated by 60% saturated ammonium sulphate, and both migrated towards the anode on electrophoresis at pH 8.6. Figueroa et al[71] suggested that both forms might be bound to protein.

Roe[66], however, has stated that all the glycogen (extractable and residual) can be removed from liver tissue by very high speed homogenisation with trichloracetic acid (TCA) using glass balls. Kits van Heijningen[313] has suggested that the need for the presence of the glass balls in the homogenisation is itself evidence for the existence of the two forms of glycogen.

Attempts have been made to extract the residual glycogen with reagents other than TCA and alkali. Lourau and Meyer found that a hot solution of 50% urea extracted the same

amount of glycogen as potassium hydroxide solution[314].
The compound obtained[315] was identified as glycogen and was contaminated with only trace amounts of phosphorus and nitrogen.

Removal of the residual glycogen by degradation of the protein by proteolytic enzymes was carried out in 1934 by Willstätter and Rohdewald[70]. Using papain and pepsin they succeeded in extracting between 30-46% of the residual glycogen, after prior extraction with TCA.

After removal of the extractable glycogen from rat liver with TCA in this present investigation, the residue was treated with pronase, a proteolytic enzyme isolated from Streptomyces griseus[316]. When the enzyme digest was subjected to gel filtration on a G-75 Sephadex column with Tris buffer, pH 8.5, the chromatographic pattern shown in Figure VIa was obtained, with the phenol-sulphuric acid positive material (A) appearing in the 30-50 ml. fraction of the eluate, followed by a major protein peak (B) detected with the Folin-Ciocalteu reagent, which was eluted between 70-250 ml. Examination of the pronase alone on Sephadex gave no peak corresponding to A (Fig. VIb) and a liver residue which had been incubated at 37° in the absence of pronase gave a possible small A peak, and a larger peak C (positive to phenol-sulphuric acid), presumably glycogen, which was eluted with the main protein

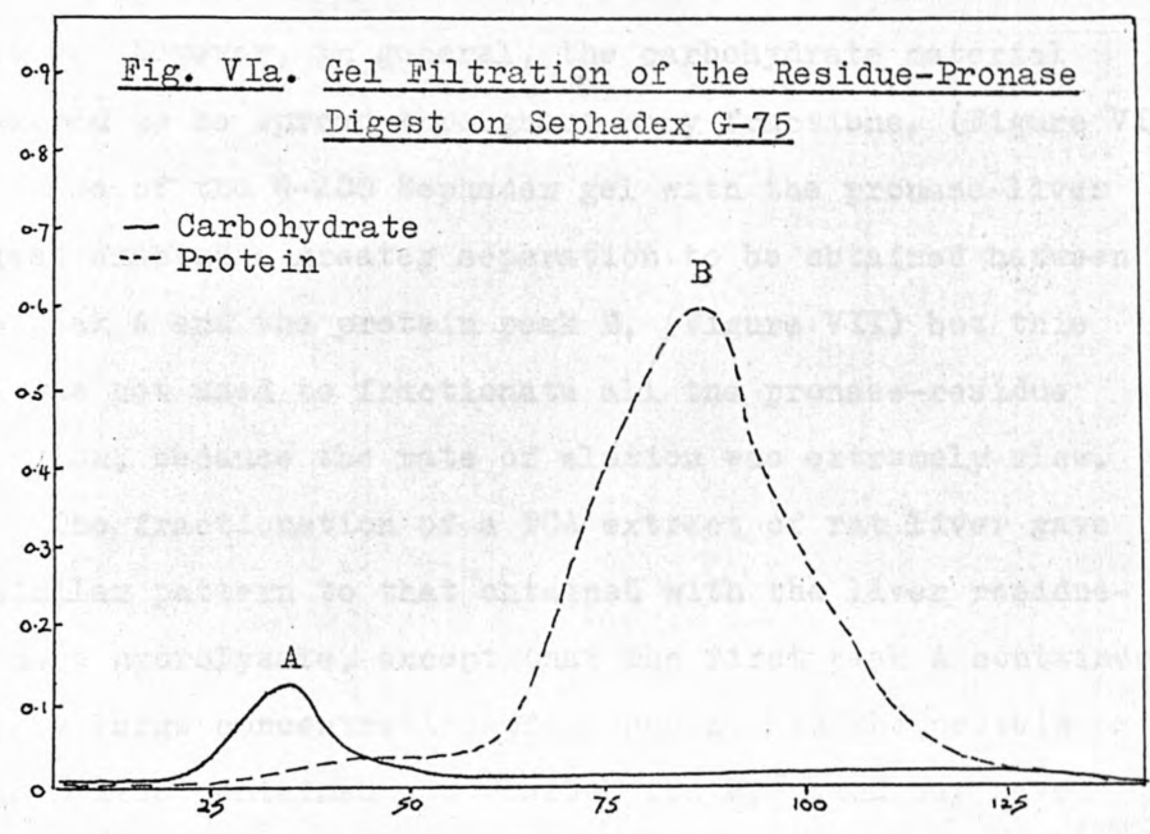


Fig. VIa. Gel Filtration of the Residue-Pronase Digest on Sephadex G-75
— Carbohydrate
--- Protein
A
B
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
25
50
75
100
125

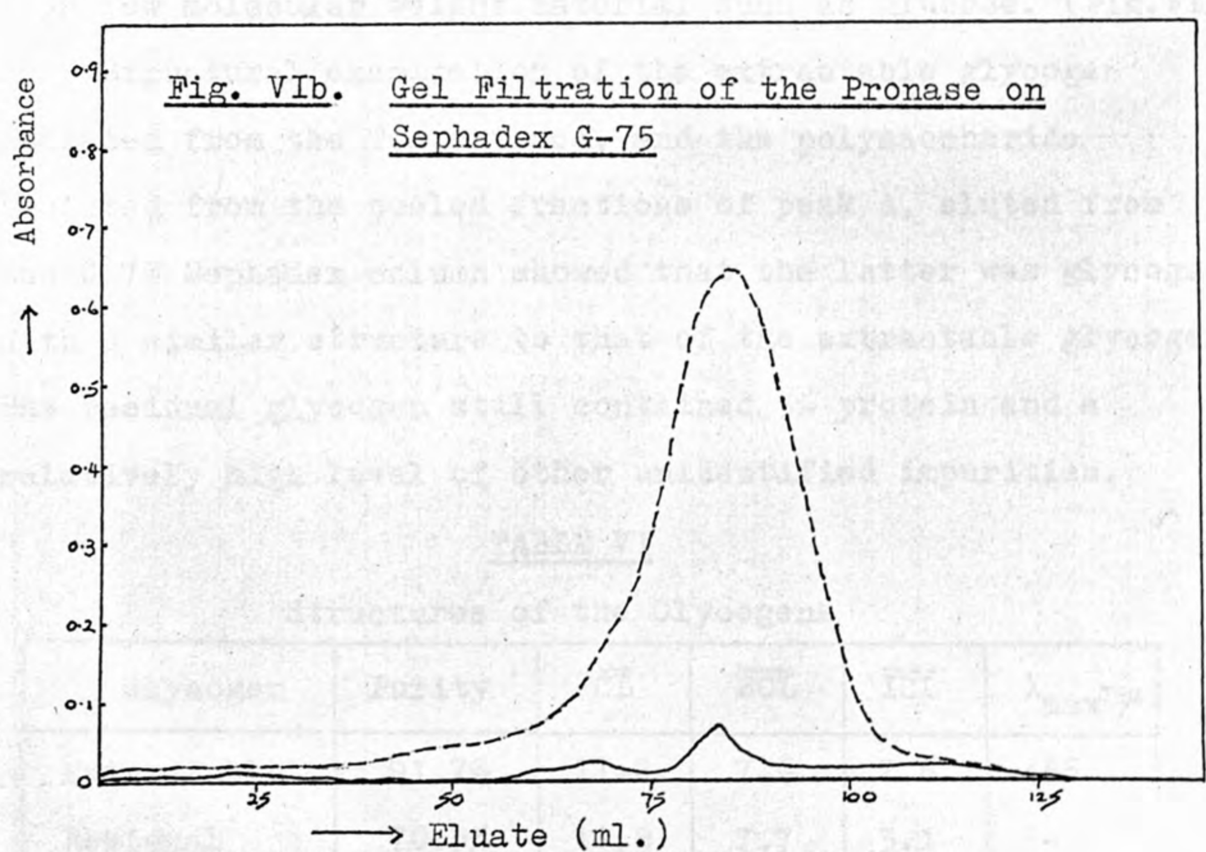


Fig. VIb. Gel Filtration of the Pronase on Sephadex G-75
↑ Absorbance
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
25
50
75
100
125
⟶ Eluate (ml.)

peak B. However, in general, the carbohydrate material appeared to be spread throughout many fractions. (Figure VIc)

Use of the G-200 Sephadex gel with the pronase-liver digest enabled a greater separation to be obtained between the peak A and the protein peak B, (Figure VII) but this gel was not used to fractionate all the pronase-residue solution, because the rate of elution was extremely slow.

The fractionation of a TCA extract of rat liver gave a similar pattern to that obtained with the liver residue-pronase hydrolysate, except that the first peak A contained a very large concentration of glycogen, and the protein peak B also contained some carbohydrate, which may have been low molecular weight material such as glucose. (Fig.VIII)

Structural examination of the extractable glycogen isolated from the TCA extract, and the polysaccharide isolated from the pooled fractions of peak A, eluted from the G-75 Sephadex column showed that the latter was glycogen with a similar structure to that of the extractable glycogen. The residual glycogen still contained 5% protein and a relatively high level of other unidentified impurities.

TABLE VI

Structures of the Glycogens

Glycogen	Purity	$\overline{CL}$	$\overline{ECL}$	$\overline{ICL}$	λ_{max} mμ
Extractable	91.7%	11.2	7.6	2.6	455
Residual	70.3%	11.8	7.7	3.1	-

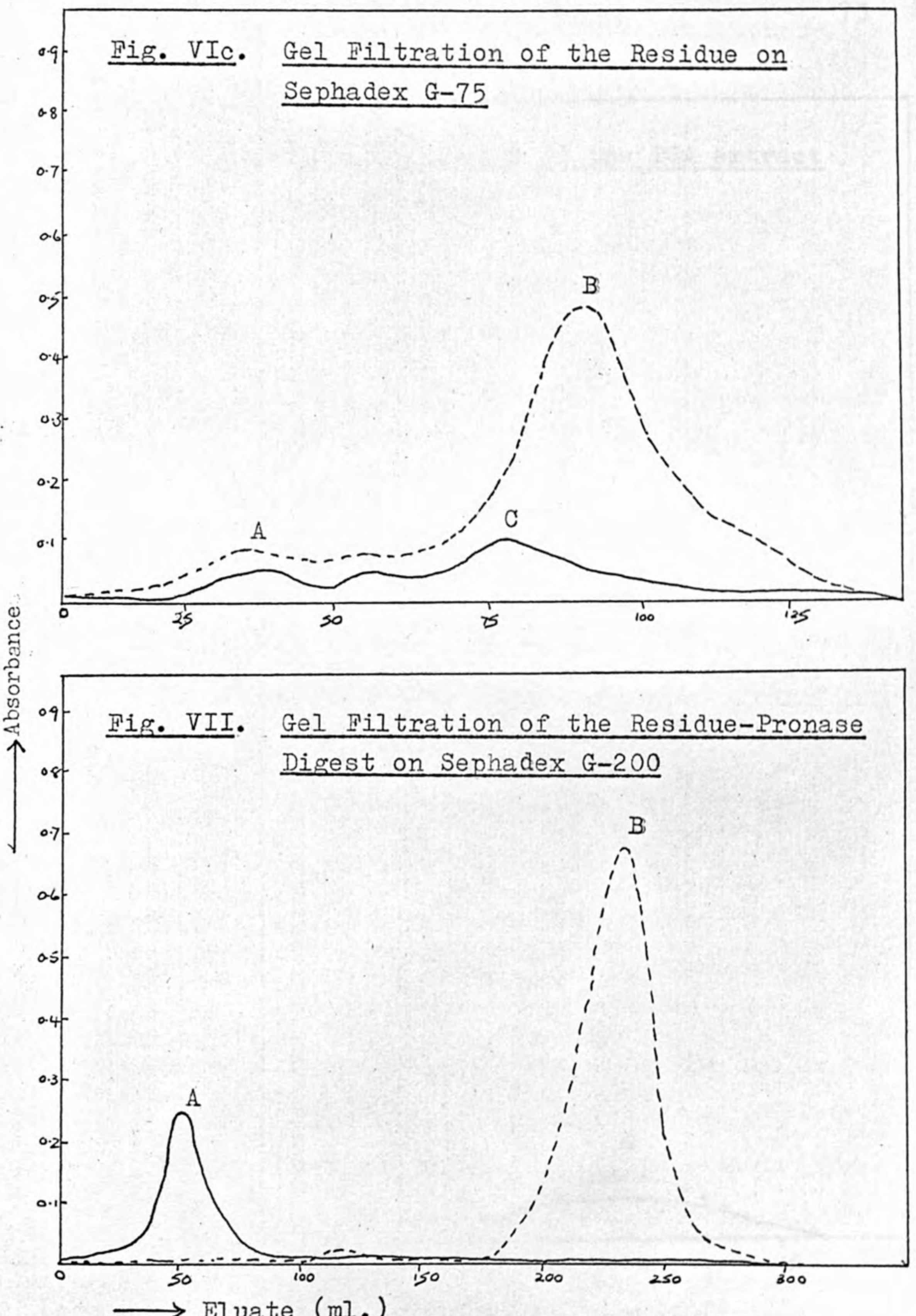


Fig. VIc. Gel Filtration of the Residue on Sephadex G-75
A
B
C
Absorbance
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
0
25
50
75
100
125
Fig. VII. Gel Filtration of the Residue-Pronase Digest on Sephadex G-200
A
B
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
0
50
100
150
200
250
300
Eluate (ml.)

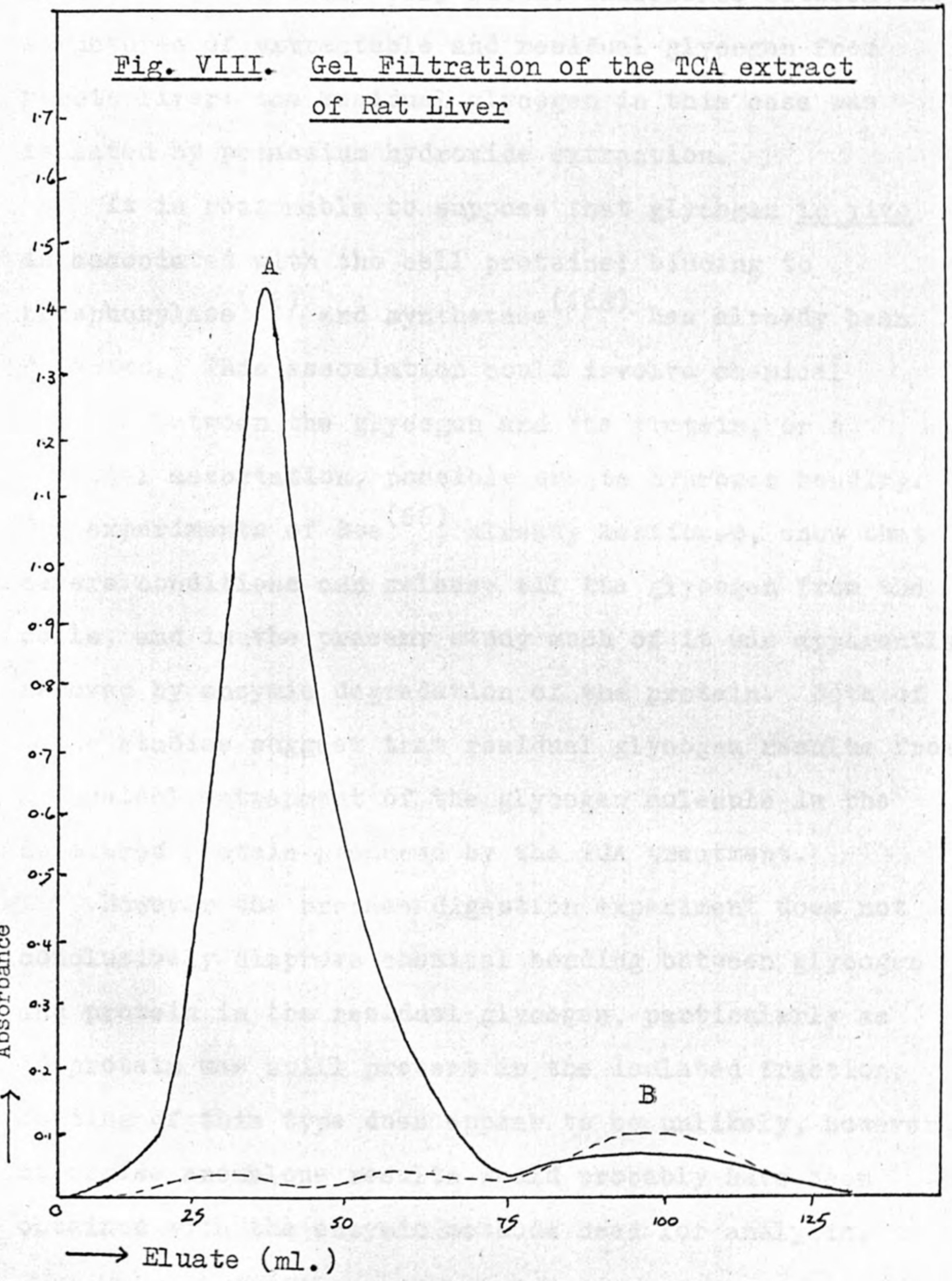


Fig. VIII. Gel Filtration of the TCA extract of Rat Liver

This confirms the work of Kjølberg, Manners and Wright[317] who found very little difference between the structures of extractable and residual glycogen from rabbit liver: the residual glycogen in this case was isolated by potassium hydroxide extraction.

It is reasonable to suppose that glycogen _in vivo_ is associated with the cell proteins; binding to phosphorylase[72] and synthetase[168] has already been detected. This association could involve chemical bonding between the glycogen and the protein, or a physical association, possibly due to hydrogen bonding. The experiments of Roe[66], already mentioned, show that severe conditions can release all the glycogen from the cells, and in the present study much of it was apparently removed by enzymic degradation of the protein. Both of these studies suggest that residual glycogen results from a physical entrapment of the glycogen molecule in the denatured protein produced by the TCA treatment.

However the pronase digestion experiment does not conclusively disprove chemical bonding between glycogen and protein in the residual glycogen, particularly as 5% protein was still present in the isolated fraction. Bonding of this type does appear to be unlikely, however, otherwise anomalous results would probably have been obtained with the enzymic methods used for analysis.

The glycogen content of the liver of fetal animals is generally found to be low during the early stages of development, but near the end of the gestational period it increases markedly to a level higher than that normally found in the adult animal[201]. After birth this glycogen store is rapidly depleted and only slowly rises again after several days.

In order to determine the variation in the quantities of extractable and residual glycogen during the fetal and neonatal period, the liver tissues from rats and humans were extracted by the method of Figueroa and Pfeiffer[318], in which the tissues were first homogenised in 5% TCA solution, and then the residue obtained from this extraction was digested with 30% potassium hydroxide solution to release the residual glycogen. The total amount of glycogen extracted with TCA and alkali, was compared with the amount of glycogen extracted using alkali alone, and good agreement in the values was obtained, confirming the observation of Koritz and Munck[319] that prolonged digestion of the tissue with alkali did not decrease the amount of glycogen.

As the quantities of fetal tissues available for analysis were very small, and also the glycogen content of these tissues was less than 1%. it was necessary to modify the normal techniques for glycogen determination. Instead of precipitating the glycogen from solution with alcohol,

TABLE VII

Glycogens in the Rat Liver

Sample	Glycogen Content in %			Ratio E/R
	Total	Extractable	Residual	
Adult				
Female I	6.14	5.31	0.83	6.14
Female II	4.82	3.94	0.88	4.47
55 hr.	0.93	0.62	0.31	2.0
48 hr.	1.09	0.61	0.48	1.26
48 hr.	1.67	1.02	0.65	1.57
24 hr.	1.59	0.82	0.77	1.07
24 hr.	0.97	0.59	0.38	1.57
16 hr.	1.32	0.74	0.58	1.24
12 hr.	1.69	1.32	0.37	3.6
6 hr.	3.72	3.24	0.48	6.98
6 hr.	3.22	2.82	0.4	6.9
4 hr.	6.56	6.13	0.43	14.2
0.5 hr.	7.52	7.05	0.47	15.0
Fetal				
20.5 days	5.22	4.83	0.39	12.45
20 days	2.84	2.49	0.35	7.1
19 days	1.16	0.80	0.36	2.22
18.5 days	1.12	0.6	0.52	1.2

the polysaccharide was estimated by direct treatment of the TCA and alkali extracts (after centrifugation) with the phenol-sulphuric acid reagent[320], after the reducing sugars in the TCA solution had been reduced by sodium borohydride[321]. This enabled very small amounts of glycogen to be measured, where isolation of the glycogen would have been impracticable if not impossible.

In fetal rats it was found that during the period from 3 days before birth until 55 hr. postnatal the amount of residual glycogen showed a small variation between 0.31 and 0.77%; the average content being 0.46%. A similar value of 0.46% was found by Russell and Bloom in adult rat liver[322]. However, during the pre- and postnatal periods mentioned, the amount of residual glycogen did increase slowly. (Figure IX and Table VII) In complete contrast to this, the amount of extractable glycogen increased 20-fold during the last two days of fetal life, and then rapidly decreased in the first 12 hours after birth. This variation in the content of extractable glycogen parallels the pattern of total glycogen storage in the liver[201]. (Figures IX and X) Gaspar[323] also noted that the livers of newborn rabbits contained a high proportion of extractable to residual glycogen, in the ratio of 4.7 ; 1.

The investigation of the amounts of extractable and

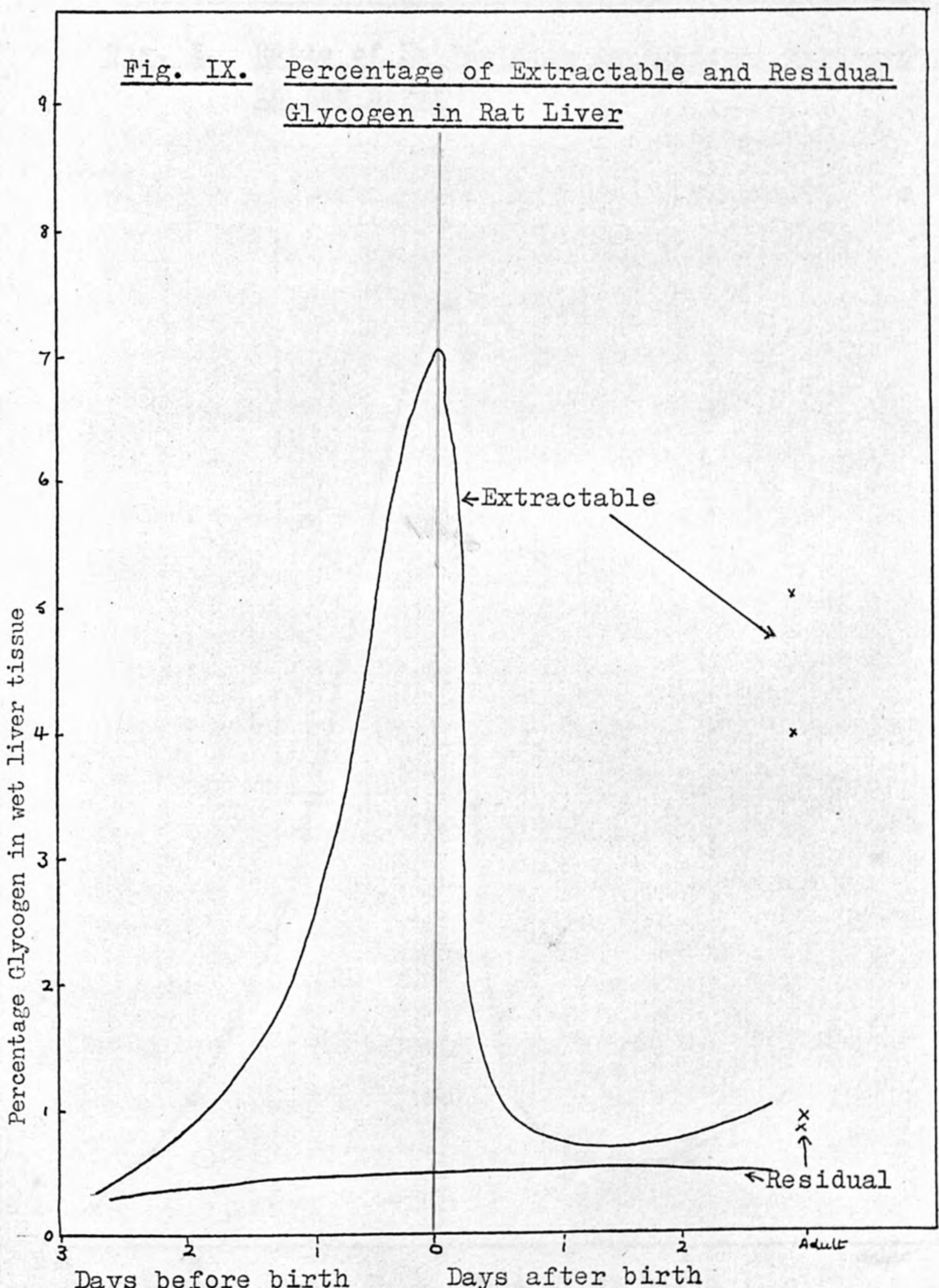


Fig. IX. Percentage of Extractable and Residual Glycogen in Rat Liver
Percentage Glycogen in wet liver tissue
9
8
7
6
5
4
3
2
1
0
3
2
1
0
1
2
Adult
←Extractable
←Residual
Days before birth
Days after birth

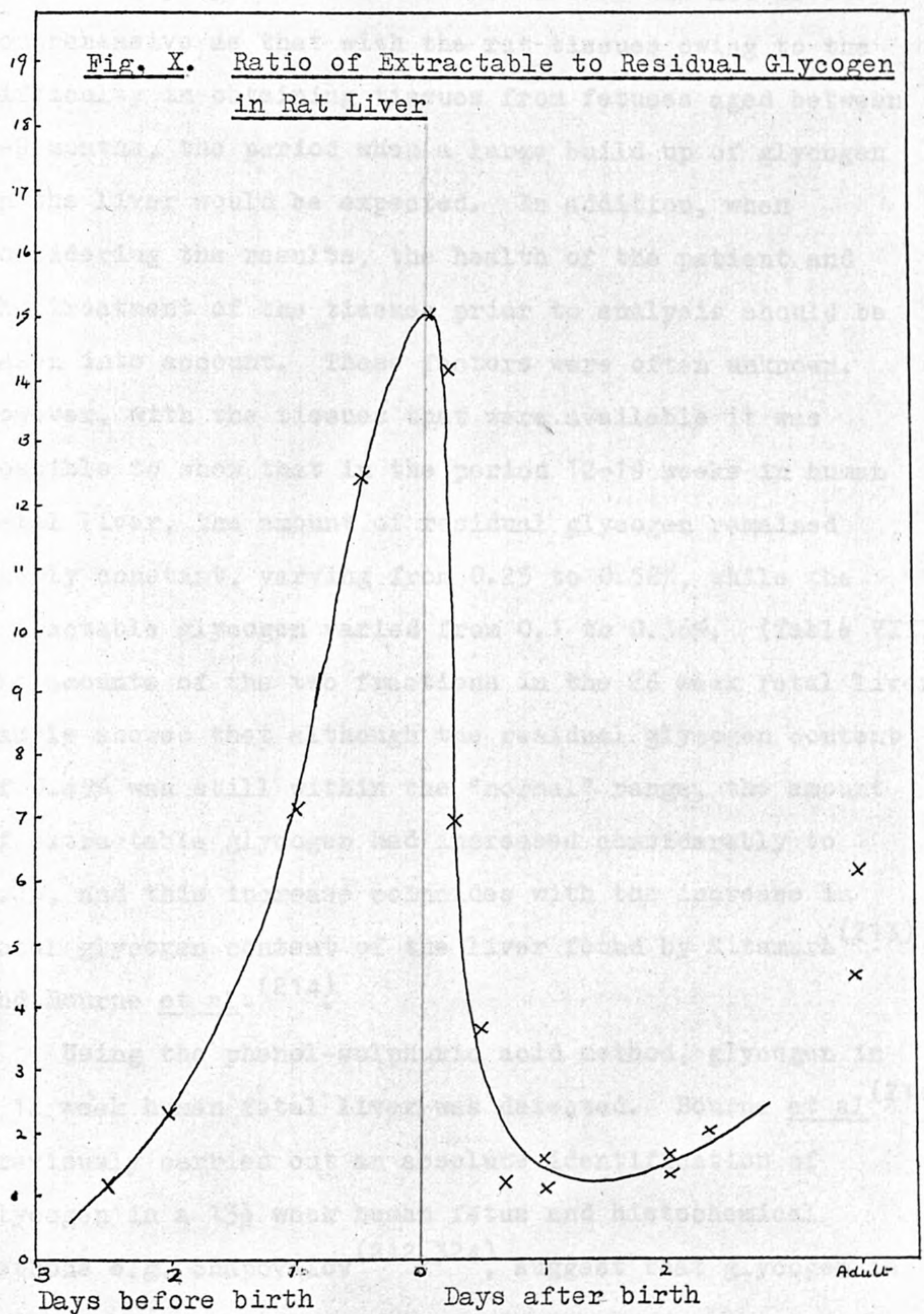


Fig. X. Ratio of Extractable to Residual Glycogen in Rat Liver

residual glycogen in human liver tissues was not as comprehensive as that with the rat tissues owing to the difficulty in obtaining tissues from fetuses aged between 7-9 months, the period when a large build up of glycogen in the liver would be expected. In addition, when considering the results, the health of the patient and the treatment of the tissues prior to analysis should be taken into account. These factors were often unknown. However, with the tissues that were available it was possible to show that in the period 12-19 weeks in human fetal liver, the amount of residual glycogen remained fairly constant, varying from 0.25 to 0.58%, while the extractable glycogen varied from 0.1 to 0.56%. (Table VIII) The amounts of the two fractions in the 26 week fetal liver sample showed that although the residual glycogen content of 0.45% was still within the "normal" range, the amount of extractable glycogen had increased considerably to 3.2%, and this increase coincides with the increase in total glycogen content of the liver found by Kitamura[213] and Bourne et al.[214]

Using the phenol-sulphuric acid method, glycogen in a 12 week human fetal liver was detected. Bourne et al[214] previously carried out an absolute identification of glycogen in a 13½ week human fetus and histochemical methods e.g. Shapovalov[212,324], suggest that glycogen is

TABLE VIII

Glycogens in the Human Liver

Sample	Glycogen Content in %			Ratio E/R
	Total	Extractable	Residual	
Adult				
12 m. 3 wk.	5.97	4.3	1.67	2.6
2 m.	6.13	5.5	0.63	8.7
1 m. 1 d.	1.34	0.68	0.66	1.1
Fetal				
26 wk.	3.65	3.2	0.45	7.1
19 wk.	0.96	0.56	0.4	1.5
18 wk.	0.47	0.12	0.35	0.33
17 wk.	0.76	0.31	0.45	0.69
16 wk.	0.71	0.21	0.49	0.43
15 wk.	1.03	0.56	0.47	1.2
14 wk.	0.95	0.37	0.58	0.63
14 wk.	0.87	0.42	0.45	0.94
14 wk.	0.47	0.22	0.25	0.83
13 wk.	0.67	0.23	0.44	0.52
12 wk.	0.37	0.1	0.27	0.35

present at even earlier stages of fetal development.

Examination of the molecular weights of liver glycogens from the fetal and adult cow showed that the molecular weight of the glycogen in the former was much lower than that of the latter.[216]

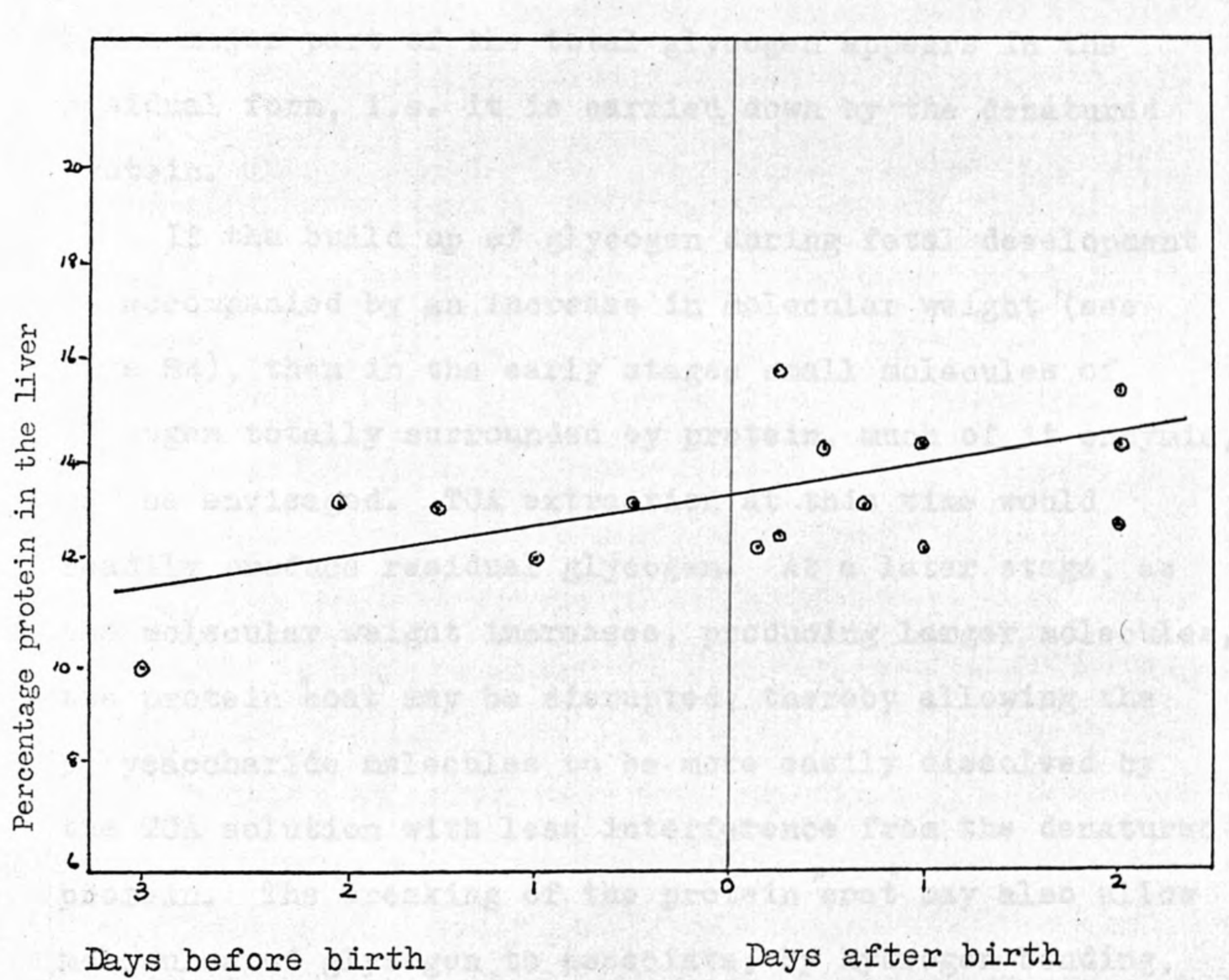
In addition, experiments with ^{14}C-labelled glucose have shown that there are two forms of glycogen differing in molecular weight and metabolic function in the rat. Stetten and co-workers[311] found that in the liver there was higher incorporation of radioactivity into the lower molecular weight fraction of TCA extracted glycogen.

Aqueous phenol extraction of rat liver has also shown that high and low molecular weight glycogen fractions exist and the former is more readily extracted than the latter.[325]

Considering these data a possible explanation for the variation of extractable and residual glycogens in liver may be attempted. Throughout development liver tissues only undergo a small increase in protein concentration (Figure XI for the rat). Glycogen, on the other hand, occurs at a low level in the early stages of gestation but towards term the synthesis of glycogen proceeds much more rapidly than that of protein and the former soon predominates. At this stage the increase in extractable glycogen may be directly related to the high

Figure XI

Protein Content in Rat Liver



glycogen : protein ratio. When this ratio decreases the percentage of extractable glycogen falls and eventually a total glycogen content is reached where there is sufficient protein to "combine" with nearly all of the glycogen and hence, when the tissues are treated with TCA a major part of the total glycogen appears in the residual form, i.e. it is carried down by the denatured protein.

If the build up of glycogen during fetal development is accompanied by an increase in molecular weight (see page 84), then in the early stages small molecules of glycogen totally surrounded by protein, much of it enzymic, may be envisaged. TCA extraction at this time would readily produce residual glycogen. At a later stage, as the molecular weight increases, producing larger molecules, the protein "coat" may be disrupted, thereby allowing the polysaccharide molecules to be more easily dissolved by the TCA solution with less interference from the denatured protein. The breaking of the protein "coat" may also allow molecules of glycogen to associate, by hydrogen bonding, to form the typical rosettes of glycogen, which have been observed with the electron microscope[326,327]

(ii) Studies on Enzymes

Paralleling studies on glycogen formation in fetal liver, the development of the activities of certain enzymes concerned with the synthesis and degradation of glycogen has also been examined. As shown in Tables IX and X and Figures XII and XIII, the activity of glycogen synthetase rises steadily during the fetal period of both rats and humans, and continues to rise after birth to levels of activity higher than that found in the adult animal. In both species the activity remains high after birth, confirming the observation of Dawkins[223]. Ballard and Oliver[228] found a similar pattern of development in sheep, the activity in the newborn animal being 210 μmoles of UDP/hr./g., compared with 181 and 55 μmoles/hr./g. for the fetus and the adult, respectively.

The development of the phosphoglucomutase activity is similar to that of synthetase, rising during gestation, and then increasing slowly after birth. The activity in human liver at birth was lower than that of a one year-old child, but the activity in the rat was somewhat higher than that found in the adult animal. A similar pattern of development was also found in sheep liver, the activity at birth being higher than in the fetus or in the mature animal[228].

The patterns of activity of the degradative enzymes have been more thoroughly investigated in an attempt to

explain glycogen storage before birth, and the rapid depletion after birth.

Phosphorylase activity has been detected early in gestation, before the deposition of glycogen, in the liver of the rat[226,227], and also in chicken liver[225]. In the present study, the activity in rats was found to be low from 16 to 19 days gestation, but then it increased up until 2 days after birth, to a level slightly above that found in the adult animal. In human liver the activity is low during the period 10 to 28 weeks, but at birth the activity is ten times greater than that found during the earlier period, and is also higher than that in a one year-old child.

The enzyme which releases glucose into the blood, glucose-6-phosphatase, was found to have very low activities during the fetal period in rats (Figure XII) and the activity only increased after birth. Jacquot and Kretchmer[328], however, found that the activity of glucose-6-phosphatase increased before birth in rats. Similar increases in activity at birth have been found in sheep[228], guinea-pigs[231] and other animals[232]. In human liver tissue aged from 10 to 29 weeks the activity of glucose-6-phosphatase varied from 0 to 62.4 µmoles P_i/ hr./g., while the activity at birth was 237 µmoles/hr./g. (Table XI, Figure XIII). Aurrichio and Rigillo[229] also

TABLE IX

Enzyme Activities of Rat Liver Tissues

Sample	Synthetase*	Phosphoglucomutase†	Phosphorylase+		Glucose-6-phosphatase+
			"a+b"	"a"	
Adult	56.6	3,585	943	-	540
Female I	-	4,915	337	284	743
Female II	70.8	2,722	440	283	310
2 d.	142	3,660	647	536	1,317
2 d.	154	4,390	712	-	1,775
2 d.	130	7,120	942	-	1,473
1 d.	132	4,380	995	960	995
1 d.	147	3,980	860	750	1,216
16 hr.	93.1	6,155	991	-	875
12 hr.	88.5	5,155	335	335	752
6 hr.	122	4,460	560	-	306
6 hr.	93.8	3,110	632	318	254
4 hr.	81.2	3,535	354	190	57.5
Fetal					
20.5 day	97.5	3,178	440	382	57.8
20 day	95.5	3,125	184	148	63.4
19.5 day	75.0	3,440	334	258	34.3
19 day	68.0	3,040	137	-	40.5
18 day	54.2	910	88.9	-	47.5
17 day	17.6	910	194	180	23.6
16 day	6.4	667	150	86.7	13.7

Activities expressed as, * = μmoles UDP/hr./g., † = μmoles G-1-P/hr./g., + = μmoles P_i/hr./g.

Figure XII

Enzyme Activities of Rat Liver Tissues

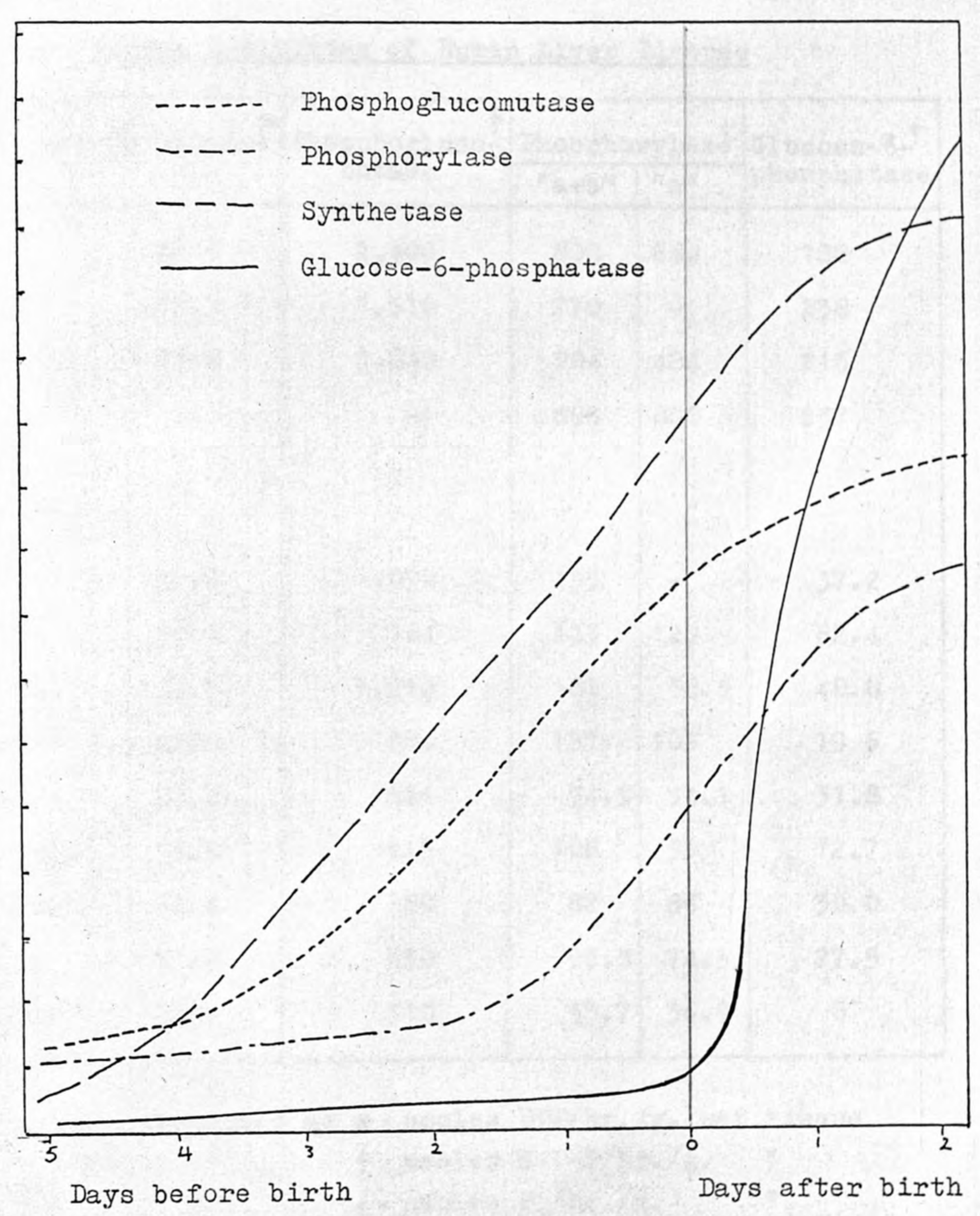


TABLE X

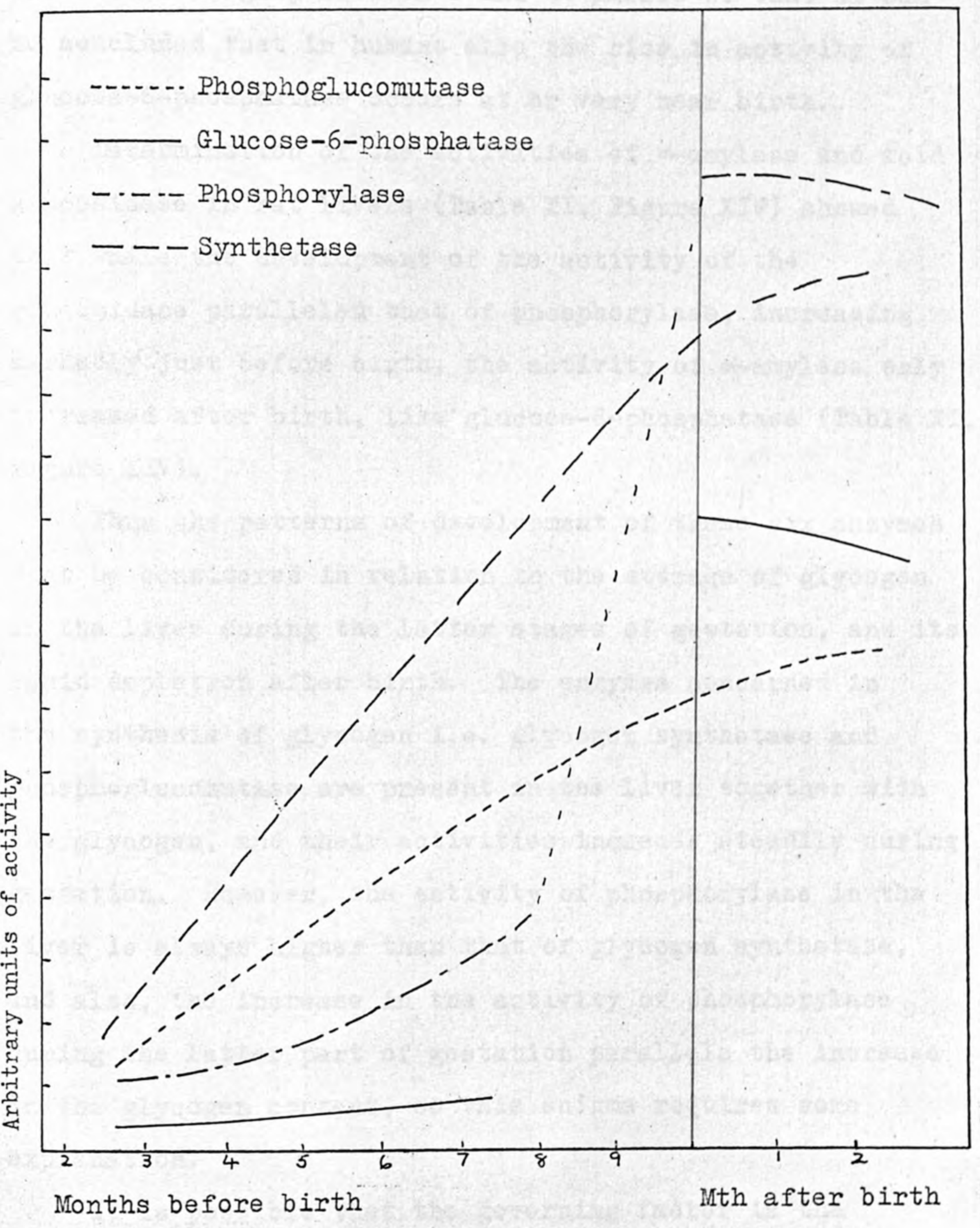
Enzyme Activities of Human Liver Tissues

Sample	Synthetase *	Phosphoglucomutase †	Phosphorylase +		Glucose-6-phosphatase +
			"a+b"	"a"	
13 m.	52.0	2,400	800	640	188
2 m.	71.5	1,310	770	-	238
1 m.	79.4	1,040	904	484	216
1 d.	-	-	895	800	237
Fetal					
29 wk.	30.0	1,090	155	-	37.2
26 wk.	19.9	744	143	120	62.4
22 wk.	37.5	1,210	181	58.5	48.8
19 wk.	44.0	880	157	103	19.6
18 wk.	27.3	501	54.5	34.1	31.8
16.5 wk.	16.6	915	106	51	12.7
14.5 wk.	21.4	780	82	56	30.0
12 wk.	25.6	630	96.5	74.5	27.5
10 wk.	11.3	310	53.7	36.9	0

Activities expressed as * = µmoles UDP/hr./g. wet tissue
† = µmoles G-1-P/hr./g. "
+ = µmoles P_i/hr./g. "

Figure XIII

Enzyme Activities of Human Liver Tissues



found low activities in fetuses aged from 20 to 39 weeks, the values ranging between 8 and 44 µmoles so that it can be concluded that in humans also the rise in activity of glucose-6-phosphatase occurs at or very near birth.

Determination of the activities of α-amylase and acid glucosidase in rat livers (Table XI, Figure XIV) showed that while the development of the activity of the glucosidase paralleled that of phosphorylase, increasing markedly just before birth, the activity of α-amylase only increased after birth, like glucose-6-phosphatase (Table XI, Figure XIV).

Thus the patterns of development of these six enzymes must be considered in relation to the storage of glycogen in the liver during the latter stages of gestation, and its rapid depletion after birth. The enzymes concerned in the synthesis of glycogen i.e. glycogen synthetase and phosphoglucomutase are present in the liver together with the glycogen, and their activities increase steadily during gestation. However, the activity of phosphorylase in the liver is always higher than that of glycogen synthetase, and also, the increase in the activity of phosphorylase during the latter part of gestation parallels the increase in the glycogen content, so this enigma requires some explanation.

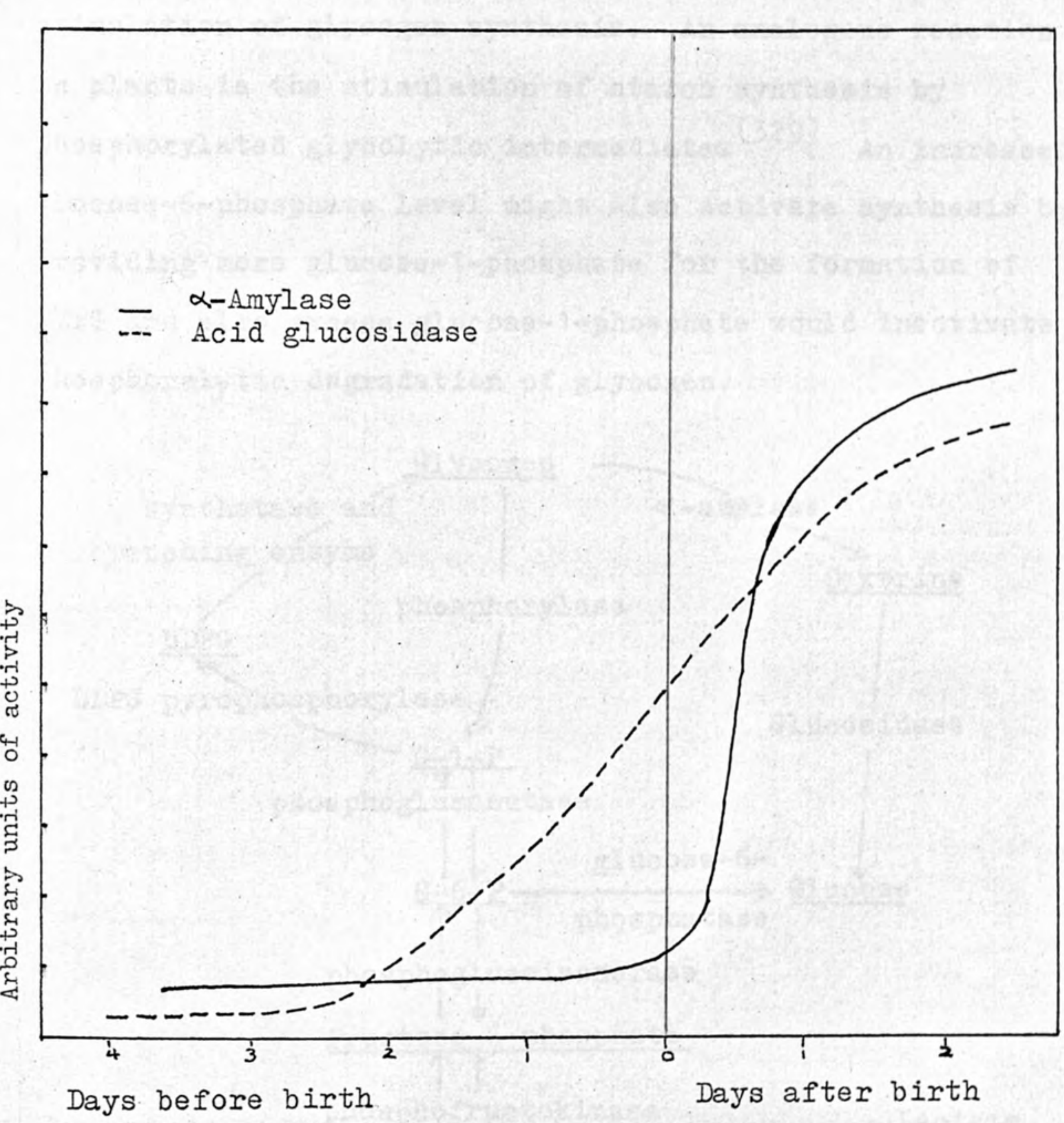
It is possible that the governing factor is the

TABLE XI

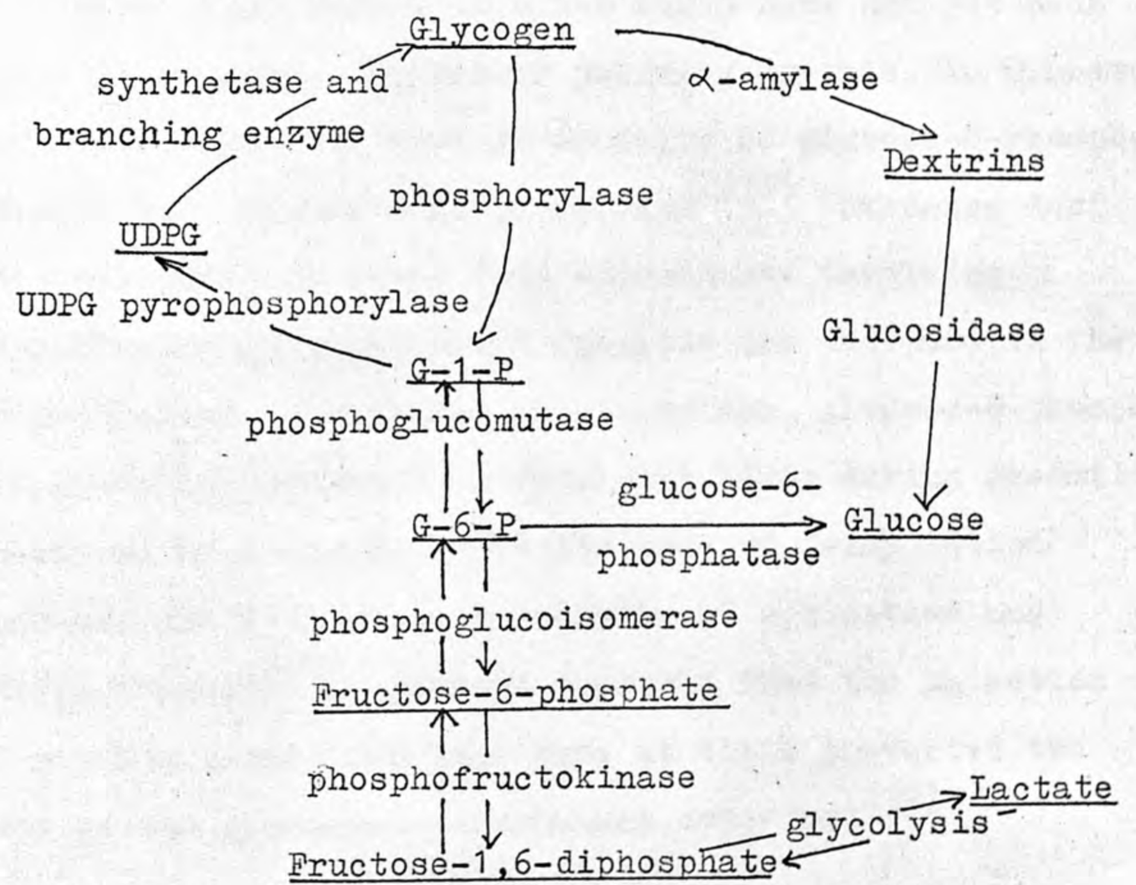
α-Amylase and Acid Glucosidase Activities in Rat Liver

Age of Sample	α-Amylase mg. equivalents of glycogen/hr./g.	Acid Glucosidase µmoles glucose/hr./g.
Adult		
Mature	11.35	56.3
2 d.	18.7	74.2
1 d.	16.7	76.7
16 hr.	14.4	47.1
12 hr.	10.9	54.4
6 hr.	5.2	56.5
6 hr.	3.2	61.4
4 hr.	3.4	63.4
Fetal		
20.5 d.	1.2	38.8
20 d.	2.0	48.9
19 d.	3.2	16.9
18.5 d.	2.9	4.3
18 d.	0.85	0
17 d.	0	4.9

Figure XIV. Activities of α-Amylase and Acid Glucosidase



glucose-6-phosphatase, which only appears just before or just after birth (Figure XII). In the absence of this enzyme a build up of glucose-6-phosphate might be expected and feed-back mechanisms could operate with the consequent stimulation of glycogen synthesis. An analogous reaction in plants is the stimulation of starch synthesis by phosphorylated glycolytic intermediates[329]. An increased glucose-6-phosphate level might also activate synthesis by providing more glucose-1-phosphate for the formation of UDPG and also excess glucose-1-phosphate would inactivate phosphorolytic degradation of glycogen.



Other enzymes besides those mentioned may affect the glucose-6-phosphate level, for example, phosphoglucoisomerase, (D-glucose-6-phosphate ketol-isomerase) or other enzymes in the glycolytic cycle. These postulated control mechanisms would presumably be disrupted at birth when the activity of glucose-6-phosphatase rapidly increases.

The pattern of α-amylase activity during gestation would also allow storage of glycogen, and the increase in activity after birth would also contribute to the degradation of the polysaccharide.

The factors which effect these increases in enzymic activities just before or after birth have not yet been fully elucidated. Premature delivery in rats, in this study, did not prevent the rise in activity of glucose-6-phosphatase, (Table XII), nor does it in rabbits[231]. Evidence for hormonal controls comes from experiments involving decapitation *in utero* which inhibits the increase in the activities of synthetase, phosphorylase, glucose-6-phosphatase and phosphoglucomutase in fetal rat liver during gestation. Treatment with cortisone at the time of decapitation prevents the reduction in activity of synthetase and phosphorylase[330]. Dawkins observed that the injection of protein inhibitors into rats at birth prevented the rise of the glucose-6-phosphatase activity.[232]

TABLE XII

Glucose-6-phosphatase Activity in Prematurely Born Rat Livers

Age of the rat	Activity (μmoles P_i /hr./g.)
0.5 hr.	98.5
4.75 hr.	195
11.0 hr.	552
25.0 hr.	980
32.0 hr.	1340
50.0 hr.	1738
55.0 hr.	1575

The time of appearance of glycogen and the enzymes in the liver during gestation depends on the animal concerned. Synthetase appears together with glycogen but before phosphorylase in the chick[224], but phosphorylase appears before the deposition of glycogen in the rat[227]. In the human fetal livers examined, (Table X, Figure XIII) the 10 week-old tissue contained glycogen, together with synthetase, phosphoglucomutase and phosphorylase, although glucose-6-phosphatase activity was not detected, so that at 10 weeks the human fetal liver is capable of synthesising glycogen. MacKay has suggested that this could occur as early as 5 weeks[331], confirming the histochemical detection of glycogen in the human fetal liver at 6 weeks[212].

(iii) Hospital Cases

Patient G.R. was found on examination at 5 months of age to have a moderately enlarged liver and slight hypoglycaemia, although no organic disease was detected. Liver function tests indicated an impairment of function as the adrenalin and glucagon responses were abnormally poor, hence suggesting glycogen storage disease. On investigation of a liver biopsy sample a glycogen content of 13% was found, confirming the diagnosis. Analysis showed that the structure of the glycogen was normal with an average chain length of 13.8 D-glucose units. The average exterior chain length by β-amylolysis was 8.9 D-glucose units, giving an average internal chain length of 3.9 units. The glycogen-iodine complex had an absorption maximum at 450 mμ, which was within the normal range.

The patient was hospitalised, and responded well to frequent feeding which prevented the reoccurrence of hypoglycaemia.

Patient P.W. had an enlarged liver, and investigation in the hospital of a biopsy sample of liver showed that phosphorylase activity was absent although debranching enzyme activity was normal. This suggested Type VI glycogen storage disease.

The liver was found to contain 18.6% glycogen, which

had an average chain length of 13 D-glucose units by α-amylolysis. The average external and internal chain lengths were 7.5 and 4.5 D-glucose units, respectively. The absorption maximum at 460 mμ confirmed that this glycogen had a normal structure.

The activity of synthetase was determined and found to be 52 μmoles UDP/hr./g. of wet tissue, which is comparable with the value of 80 μmoles UDP/hr./g. found in normal liver by Larner[332].

Patient B.W.J. suffered from pain and stiffness in the muscles after exercise, and a diagnosis of Type V glycogen storage disease was proposed.

The activities of phosphorylase, phosphoglucomutase and glycogen synthetase were measured on a muscle biopsy specimen, as shown in Table XIII.

TABLE XIII

Enzymic Activities in Patients B.W.J. and P.K.B.

Enzyme *	B.W.J.	P.K.B.	Normal Value[332]
Phosphoglucomutase	5,730	6,030	5950
Phosphorylase, + AMP	236	289	117-600
Phosphorylase, - AMP	197	139	56
Synthetase	-	78	14.3-45.1

* Activity expressed in μmoles / hr. / g. wet tissue.

Although the activities of phosphorylase and phosphoglucomutase are normal, the lack of synthetase activity in the sample from B.W.J. was probably due to loss of enzyme during the removal and storage of the tissue prior to determination.

Patient P.K.B. aged 15 years also suffered severe cramp after exercise. Examination of a muscle biopsy specimen (Table XIII) showed that the phosphorylase and phosphoglucomutase activities were normal, whilst that of synthetase was somewhat higher than the values found by Larner[332].

Thus the analyses showed that neither B.W.J. nor P.K.B. suffered from Type V glycogen storage disease, but examination of the muscle tissue of B.W.J. by electron microscopy showed that the symptoms were due to a gradual degeneration of the muscle tissue rather than glycogen storage disease.

General Methods

Chromatography

Whatman No. 1 paper was used throughout with the following solvent systems:

1. Ethyl acetate: acetic acid: water[333] 9:2:2, v/v
2. Acetone: n-butanol: water[334] 5:3:2, v/v

The sprays used for locating carbohydrates were:

1. p-Anisidine hydrochloride (2%, w/v) in n-butanol[335].
2. Silver nitrate solution in acetone, followed by ethanolic sodium hydroxide[336].

EXPERIMENTAL

[illegible]etric Methods

Reducing sugars were determined by the Park and Johnson method[337], unless otherwise stated.

Phosphate was measured by the method of Fiske and Subbarow[338].

Apparatus

Absorbances of coloured solutions were measured using either a Hilger "Spekker" or a Unicam S.P.500 spectrophotometer. Ultraviolet spectra were obtained using a Perkin-Elmer 137 UV recording spectrophotometer.

Infrared spectra were measured using a Perkin-Elmer Infracord spectrophotometer.

General Methods

Paper Chromatography

Whatman No. 1 paper was used throughout with the following solvent systems:

A Ethyl acetate: acetic acid: water[333] 9:2:2, v/v

B Acetone: n-butanol: water[334] 5:3:2, v/v

The sprays used for locating carbohydrates were:

I p-Anisidine hydrochloride (2%, w/v) in n-butanol[335].

II Silver nitrate solution in acetone, followed by ethanolic sodium hydroxide[336].

Colorimetric Methods

Reducing sugars were determined by the Park and Johnson method[337], unless otherwise stated.

Phosphate was measured by the method of Fiske and Subbarow[338].

Apparatus

Absorbances of coloured solutions were measured using either a Hilger "Spekker" or a Unicam S.P.500 spectrophotometer. Ultraviolet spectra were obtained using a Perkin-Elmer 137 UV recording spectrophotometer.

Infrared spectra were measured using a Perkin-Elmer Infracord spectrophotometer.

Experiment 1. Extraction of glycogen.

The glycogen was extracted from frozen samples of liver by pulverising the tissue in 10% trichloracetic acid solution[66]. The homogenate was centrifuged at 3,000 r.p.m. for 10 min. at 0°, and the supernatant liquid was decanted off. The precipitate was re-extracted three times with 10% trichloracetic acid and the final residue was discarded.

The supernatants were pooled and 5 volumes of ethanol added, and after standing overnight at 4° the glycogen settled and was collected by centrifugation, then washed twice with ethanol and finally with ether. The glycogen was dried over phosphorus pentoxide for several days, and the final traces of moisture were removed by drying under reduced pressure at 65° for three days.

Experiment 2. Determination of the purity of glycogen.

Glycogen (1 mg.) was hydrolysed to glucose by heating for 2 hr. at 98° in 2N-sulphuric acid (1 ml.)[339]. The solution was cooled, neutralised with 2N-sodium hydroxide and diluted with water to 5 ml. The amount of glucose produced was measured using either the Biochemica "Glucose Reagent" (Boehringer, Mannheim, Germany.) or the Park and Johnson method; the absorption of the blue colour produced was measured with a Hilger "Spekker" using an Ilford No. 609 filter.

Experiment 3. The infrared spectra of glycogen.

The spectrum of glycogen from 650-4,000 cm^{-1} was obtained using a potassium bromide disc containing 2% polysaccharide.

Experiment 4. Determination of the activity of -amylase.

The α-amylase used was a sample extracted from human saliva[340] by Dr. McLean, and freeze-dried in 100mM-phosphate-citrate buffer, pH 7,0.

The method of Manners and Wright[46] was followed; they defined the unit of activity of α-amylase as that quantity of maltose liberated from 25 ml. of 1% starch solution in 3 min. at 35° by 1 mg. of enzyme.

An "Analar" soluble starch solution (1%, 25 ml.) was incubated with the freeze-dried sample of α-amylase (0.1 mg. in water (2 ml.)) in the presence of 0.05% sodium chloride (3 ml.) for 30 min. at 37°. The digests were diluted to 50 ml. with water and the amount of maltose liberated was determined. As a check, further incubations were carried out using half the previous amount of enzyme (0.05 mg. in 2 ml. of water) to ensure that an excess of enzyme had not been used.

Sample	Amount of Enzyme	Mg. maltose produced	Activity
I	0.08 mg.	36.5	46
II	0.10 mg.	44.0	44
III	0.05 mg.	22.5	45

The activity of this sample of α-amylase was 45 units per mg. of enzyme.

Experiment 5. Incubation of α-amylase with maltose.

This experiment was carried out to determine whether there was any maltase contamination of the α-amylase[46].

To chromatographically homogeneous maltose (5 mg.), α-amylase [1 mg. in water (1 ml.)] and 0.05% sodium chloride (1 ml.) were added and the solution was diluted to 10 ml. with water. The solution was incubated for 48 hr. at 37° and at hourly intervals (from 1-8 and 22-29 hr.) samples were removed, deionised with "Biodeminrolite" and examined by paper chromatography using solvent A, the spots being located using spray I.

After incubation for 48 hr. the residual solution was deionised and concentrated under reduced pressure and examined as before. No trace of glucose was found at any stage of the incubation indicating that there was no maltase activity present.

Experiment 6. Investigation of the reaction path of α-amylase on glycogen.

Glycogen (1 mg.) was incubated at 37° with α-amylase (0.1 mg. in 1 ml. of water, i.e. 4.5 units[339]), 0.05% sodium chloride (1 ml.) and water to 10 ml. At intervals the amount of reducing sugars produced was determined. It was found that after 6 hr. the production of reducing sugars and hence the percentage hydrolysis (Pm) were constant. A 6 hr. incubation period was therefore used in later work.

Percentage hydrolysis of rabbit liver glycogens.
(Experiment performed in duplicate)

Time	0.25 hr.	1 hr.	3 hr.	5 hr.	6.5 hr.
Pm %	39	65	73	70	73
Time	0.75 hr.	3 hr.	3.5 hr.	4.5 hr.	7 hr.
Pm %	57.5	70	76.5	77.5	76.5

Experiment 7. Determination of the average chain length of glycogen using α-amylase.

Following the method of Manners and Wright[46], glycogen (1 mg.) was hydrolysed with α-amylase (4.5 units per mg. glycogen[339]), with 0.05% sodium chloride (1 ml.) as an activator, and water to 10 ml. After incubation for 6 hr. at 37° the amount of "apparent" maltose produced was determined.

Paper chromatographic analysis showed that maltose and glucose were the main products of hydrolysis.

Experiment 8. Determination of the activity of β-amylase.

The β-amylase used was a solid preparation from the Wallerstein Company, Mariners Harbor, New York.

The activity was determined by the method of Hobson, Whelan and Peat[341], who defined the activity of the enzyme as the quantity of maltose liberated by 1 mg. of enzyme under the following conditions.

An "Analar" soluble starch solution (0.6%, 25 ml.) was incubated with 0.2M-sodium acetate buffer (pH 4.8, 3 ml.) and the enzyme solution (40 mg. in 2 ml. of water) for 30 min. at 32°. The digest was diluted with water to 50 ml. and the amount of maltose produced was determined.

To check that excess enzyme had not been used the incubations were repeated using half the previous quantity of enzyme.

Sample	Amount of Enzyme	Mg. maltose produced	Activity
I	18.8 µg.	3.75 mg.	200
II	18.8 µg.	4.0 mg.	213
III	37.6 µg.	7.25 mg.	193

The activity of β-amylase was 202 units per mg. of enzyme.

Experiment 9. Investigation of the reaction path of β-amylase.

Glycogen (26 mg.) was incubated at 37° with β-amylase solution (13 mg. in 1 ml. of water), 0.2M-sodium acetate buffer, pH 4.6 (5 ml.) and water to 25 ml.[339] At regular intervals samples (2 ml.) were removed and the amount of maltose produced was determined using the Somogyi method[342]. It was found that after 18 hr. up to 24 hr. the maltose production remained fairly constant, but after 35 hr. slow hydrolysis, presumably due to α-amylase impurities, began to occur. Thus 24 hr. was selected as a suitable incubation period.

The reaction pattern using calf liver glycogen.

Time	7.5 hr.	24 hr.	33 hr.	48 hr.	72 hr.
Hydrolysis	38%	40.5%	40.2%	42%	44.5%

Experiment 10. Determination of the external chain length of glycogen.

To glycogen (1-2 mg.) dissolved in water (1 ml.), was added 0.2M-sodium acetate buffer, pH 4.6 (0.4 ml.) and β-amylase solution (1 ml.) equivalent to 40-50 units per mg. glycogen[47]. The solution was diluted to 10 ml. with water and incubated for 24 hr. at 37°, when the amount of maltose produced was determined. From the percentage

β-amylolysis the average number of glucose units removed during the hydrolysis was calculated. The factor 2.5 was added[304] to correct for the number of glucose units left attached at the branch point, and thus the average external chain length ($\overline{ECL}$) of the glycogen was determined.

The internal chain length ($\overline{ICL}$) is given by:

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

The only reducing sugar present in the reaction mixture was shown to be maltose by paper chromatography.

Experiment 11. Preparation of a β-amylase limit dextrin of glycogen.

Glycogen (100 mg.) was incubated with β-amylase (40-50 units per mg. glycogen), 0.2M-sodium acetate buffer, pH 4.6 (4 ml.) and water to 100 ml. for 24 hr. at 37°. The maltose produced was estimated to check that the β-amylolysis was complete.

The solution was dialysed overnight against running water and then reduced in volume by evaporation under reduced pressure. The protein was removed by shaking for 1 hr. with 0.25 volumes of chloroform and 0.1 volumes of *iso*-amyl alcohol[307]. The final traces of amyl alcohol were removed by washing the aqueous layer three times with ether. The aqueous layer was dialysed again against water and concentrated as before.

The limit dextrin was precipitated by the addition of 5 volumes of ethanol, and, after standing overnight at 4°, was collected by centrifugation, washed three times with ethanol and finally with ether. The sample was dried at 60° under reduced pressure in the presence of phosphorus pentoxide.

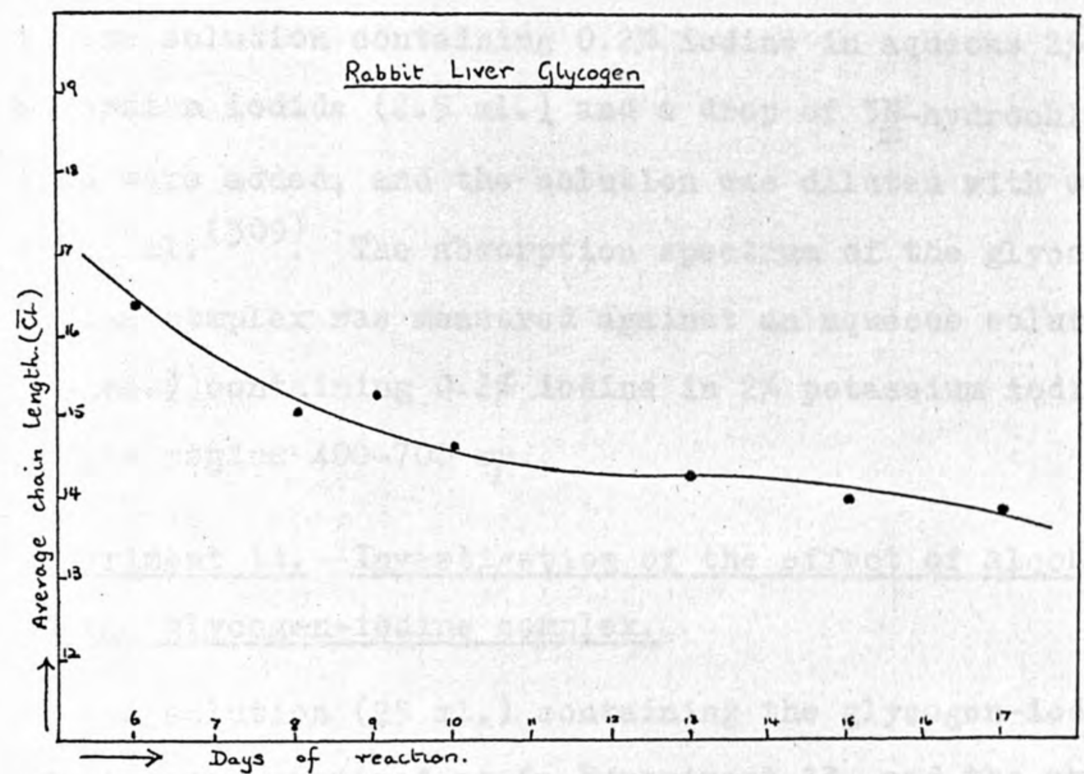
The structure and purity were determined as with glycogen.

<u>Experiment 12.</u> <u>Determination of the average chain length of glycogen using periodate oxidation.</u>

Glycogen (50-100 mg.) was dissolved in carbonate-free water (2 ml.), and after the addition of 8% (w/v) sodium metaperiodate (3 ml.) the solution was diluted with water to 25 ml.[343] A blank was similarly prepared omitting the glycogen. The two solutions were kept in the dark at 4° and at two day intervals after the 5th. day samples (2 ml.) were removed and ethylene glycol (0.1 ml.) was added to remove the excess metaperiodate. The formic acid liberated during the oxidation of the glycogen was titrated rapidly against 0.01N-sodium hydroxide delivered from an "Agla" microburette, using methyl red as indicator.

All the water used in the experiment was made carbonate-free by boiling and the operations to determine the formic acid were performed as quickly as possible to prevent the absorption of carbon dioxide from the atmosphere.

The blank compensated for any acid reaction due to the ethylene glycol, which was found to be slight but not negligible.



After 10-12 days the amount of formic acid produced was constant but this was followed by a period of slow over-oxidation.

Incubations were also attempted at 25° and 35° in order to curtail the time needed for reaction, but this resulted in marked over-oxidation.

Experiment 13. The absorption spectra of polysaccharide-iodine complexes.

To glycogen (2-5 mg.) dissolved in water (2 ml.), iodine solution containing 0.2% iodine in aqueous 2% potassium iodide (2.5 ml.), and a drop of 3N-hydrochloric acid were added, and the solution was diluted with water to 25 ml.[309] The absorption spectrum of the glycogen-iodine complex was measured against an aqueous solution (25 ml.) containing 0.2% iodine in 2% potassium iodide (2.5ml.) in the region 400-700 mμ.

Experiment 14. Investigation of the effect of alcohols on the glycogen-iodine complex.

A solution (25 ml.) containing the glycogen-iodine complex was prepared as in Experiment 13, and the absorption spectrum in the region 400-700 mμ was measured on a recording UV spectrophotometer. Methanol (2 ml.) was added and the the solution throughly mixed. The absorption of the resulting solution was measured as before against water as the blank.

The procedure was repeated with ethanol and 1-propanol.

Experiment 15. Investigation of the effect of alcohols on an aqueous solution of iodine and potassium iodide.

To an aqueous solution (25 ml.) containing 0.02% iodine, 0.2% potassium iodide and a drop of 3N-hydrochloric acid, methanol (2 ml.) was added and the solution was throughly mixed. Other solutions were similarly treated with ethanol and 1-propanol. Absorption spectra of these solutions were measured against water as in Experiment 14.

A solution of iodine and potassium iodide was similarly diluted with water (2 ml.) and the absorption spectrum measured.

To determine the effect of the added alcohol on the $I_3^{\ominus}$ ion, the absorption spectrum in the region 250-400 mμ was measured for a solution containing 0.02% iodine and 0.2% potassium iodide in ethanol, and a similar solution, to which water (0.25 ml.) had been added. Acid was not added to these solutions.

Experiment 16. Determination of the effect of the protein extraction procedure on the glycogen-iodine complex formation.

The absorption spectrum in the region 400-700 mμ of the iodine complex of rabbit liver glycogen II was measured. A sample of the same glycogen was dissolved in water and treated with the Sevag reagent[307] (Expt. 11) After isolating the glycogen and drying it under reduced

pressure over phosphorus pentoxide, the absorption spectrum of the glycogen-iodine complex was re-examined.

A sample of this extracted glycogen was dissolved in water and extracted three times with ether. The glycogen was isolated, dried and the spectrum of the iodine complex again examined.

Experiment 17. Determination of the effect of urea on the glycogen-iodine complex.

Solutions (25 ml.) of glycogen (3 mg.), iodine, potassium iodide and acid were made up as in Experiment 13, containing 0, 2, 4, 6 and 8M-urea. The absorption spectra of these solutions were measured against blanks containing the same amount of urea, iodine and potassium iodide. The pH of these solutions was also measured.

To determine the effect of urea on the solution of iodine in aqueous potassium iodide, the absorption of the "blank" solutions containing 0.2% potassium iodide, 0.02% iodine and 2, 4, 6 and 8M-urea were measured against an aqueous solution containing 0.1% potassium iodide and 0.01% iodine.

Three solutions were prepared containing 0, 2 and 8M-urea, to which were added aliquots (0.2 ml.) of the aqueous 2% potassium iodide-0.2% iodine solution, and after the addition of one drop of 3N-hydrochloric acid, water was added to 25 ml. The absorption spectra of these solutions were measured against water as a blank.

Experiment 18. Preparation of pullulanase solution.

The sample of crude pullulanase (110 mg.) was received as a gift from Professor W. J. Whelan.

A portion of the solid (52.9 mg.) was gently shaken overnight in 20mM-phosphate buffer, pH 6.8 (2 ml.). The solution was centrifuged at 15,000 r.p.m., and the supernatant dialysed for 24 hr. at 0° against the same phosphate buffer. The solution was transferred to a 10 ml. flask and stored at 4°. All operations during the extraction procedure were carried out at 4° [344].

Experiment 19. Incubation of β-amylase and glucamylase limit dextrins of glycogen with pullulanase.

Glycogen β-amylase limit dextrin (0.68 mg.) and glucamylase limit dextrin (0.79 mg.) [samples obtained by Dr. McLean] were dissolved separately in 0.2M-citrate buffer, pH 5.0 (0.1 ml.) and to each sample pullulanase solution (0.1 ml.) was added[344]. The reaction tubes were incubated at 32° for 2 hr.. Samples were removed and examined on paper chromatograms using solvent A, and the sugars were located with reagent I.

The digests were incubated for a further 2 hr. at 32° and after deionising with Biodeminrolite, were examined as before.

Examination of the products of enzymic hydrolysis by electrophoresis in borate complexing reagent at pH 9.8

on a "Shandon" High Voltage Electrophoresis apparatus was unsuccessful.

Experiment 20. Incubation of glycogen with pullulanase and β-amylase alternately.

Rabbit liver glycogen II (15.2 mg.) was dissolved in water (1 ml.) and pullulanase solution (0.5 ml.) and 0.2M-citrate buffer, pH 5.0 (2 ml.) were added. On dilution with water to 10 ml. the digest (Solution I) was incubated at 37° for 15 hr. The solution was boiled for 15 min. to denature the pullulanase and then cooled. An aliquot (1 ml) was removed and to this β-amylase [114 units in 0.2M-sodium acetate buffer, pH 4.5 (1 ml.)] and water (8 ml.) were added (Solution II). The reaction mixture was incubated at 37° for 24 hr. and the β-amylase then deactivated by boiling for 15 min. After cooling, the reducing sugar produced was measured.

This whole procedure was repeated twice with Solution II using the same amounts of the enzymes.

Experiment 21. Recovery of TCA-extractable glycogen from rat liver.

Liver tissue (26 g.) from two rats was homogenised in ice-cold water (200 ml.) and the homogenate was centrifuged at 600g for 15 min. to remove gross tissue debris[71]. The residue was discarded, and the supernatant

solution after treatment with an equal volume of 10% trichloracetic acid solution (TCA), was left to stand for 15 min. The solution was centrifuged and the centrifugate (Solution A) decanted off. The residue was washed three times by gentle homogenisation with cold 5% TCA solution (20 ml. portions) and the washings were added to solution A.

Five volumes of ethanol were added to solution A, and after standing overnight at 4° the precipitated glycogen was removed by centrifugation, washed with ethanol and ether and dried. This, the TCA-extractable glycogen, was subjected to structural analysis.

The precipitate resulting from the trichloracetic acid treatment was suspended in water (20 ml.) and dialysed against running water for 24 hr. The dialysed solid, the TCA-residue, was filtered off and stored at -20°.

Experiment 22. Treatment of the TCA-residue with pronase.

Pronase was obtained from Calbiochem.

A sample of the TCA-residue (5.7 g.) was homogenised in 50m$\underline{\underline{M}}$-phosphate buffer, pH 7.4 (100 ml.) and pronase (200 mg.) was added. The digest was incubated at 37° for 96 hr. and was then stored until use at -20° (345).

Control solutions containing (i) TCA-residue (2 g.) in buffer (50 ml.) and (ii) pronase (50 mg.) in buffer (50 ml.) were similarly incubated, and then stored at -20°. Thymol

was added to all digests to inhibit bacterial growth.

Glycogen (6.8 mg.) was incubated at 37° with pronase (4.4 mg.) in the phosphate buffer (25 ml.). At intervals the reducing power was measured.

Time	35 min.	6.25 hr.	24 hr.
Reducing Power	1.62%	1.69%	1.69%

After thawing, a sample (10 ml.) was removed from the solution containing the TCA-residue and pronase, and after centrifugation, the supernatant was subjected to gel filtration on a column (3.5 x 8 cm.) of Sephadex G-75, which had previously been equilibrated with 10m$\underline{\underline{M}}$-Tris buffer, pH 8.5. Elution was carried out with the same buffer and the fractions (5 ml.) collected were checked for absorption at 260 and 280 mμ, and for protein and carbohydrate[346,320].

Samples (10 ml.) from the two controls were similarly treated on the same Sephadex column, the eluted fractions being analysed as before.

A second digest of the TCA-residue (3 g.) and pronase (100 mg.) in phosphate buffer (50 ml.) was incubated at 37° for 96 hr., together with a control digest containing only the TCA-residue (2 g.) in buffer (50 ml.). Samples (5 ml.) from these solutions were, after centrifugation, subjected to gel filtration on a longer column (3.5 x 15 cm.)

of Sephadex G-75 (Column II), and the eluted fractions were analysed as before.

Experiment 23. Preparative separation of carbohydrate on the Sephadex column.

The remaining TCA-residue was treated with pronase as described in Experiment 22 and samples (10 ml.) fractionated on the Sephadex column II. The fractions of the first peak from all the fractionations were combined. The solution was concentrated by evaporation under reduced pressure, and the concentrated solution (10 ml.) was treated with 10% TCA solution (3 ml.). The precipitate was removed from the solution by centrifugation and then filtering, and the filtrate was concentrated as before. This solution was treated with 5 volumes of ethanol, and then allowed to stand overnight at 4° to precipitate the polysaccharide. This precipitate was isolated by centrifugation, washed twice with ethanol and finally with ether. After drying structural analysis was carried out.

Experiment 24. Examination of the TCA-residue digest on a Sephadex G-200 column.

To obtain a better separation of the components found in the first digest of the TCA-residue with pronase, (Experiment 22) a sample (5 ml.) was centrifuged and the supernatant solution was subjected to gel filtration on

a column (2.5 x 40 cm.) of Sephadex G-200 equilibrated with 10mM-Tris buffer, pH 8.5. The elution and analysis were carried out as in Experiment 22.

Experiment 25. Examination of a liver extract on Sephadex.

Rat liver (500 mg.) was homogenised in 3% TCA solution (5 ml.) and the solution was centrifuged. The supernatant (4 ml.) was subjected to gel filtration on the Sephadex G-75 column I, as described in Experiment 22.

Experiment 26. Determination of the amount of extractable and residual glycogen in liver tissue

Liver tissue (100-600 mg.) was pulverised in 5% tri-acetic acid solution and centrifuged. The supernatant was decanted off and the residue was gently washed with the TCA solution, the washing being added to the supernatant.

After neutralising the supernatant solution with sodium hydroxide, sodium borohydride (5-10 mg.) was added to reduce all the free glucose[321], and the solution was diluted with water to 50 ml., and placed in an ice-bath for 45 min.

The residue was digested in 30% potassium hydroxide (3 ml.) at 98° for 45 min. After cooling the solution was diluted to 25 ml. with water.

Samples (0.5 ml.) were taken from both solutions and the amount of polysaccharide present was determined using the phenol-sulphuric acid method[320]. A blank containing

only the sample of residue digest and sulphuric acid was also prepared to check the amount of colour produced by the sulphuric acid reacting with the digest.

Neutralisation, or acidification of the digest before dilution had no effect on the amount of colour produced during the estimation procedure.

To check the method used above a sample of rat liver (0.77 mg.) was homogenised in ice-cold water (5 ml.) and two samples (2 ml.) were removed. One was treated as described above while the other was digested with sodium hydroxide (5 ml.). The amounts of polysaccharide produced by the two methods was compared.

TCA	KOH_R	TOTAL	KOH_W
5800γ +	1300γ =	7100 γ	7000 γ

Experiment 27. Determination of the water content of liver tissue.

Known weights of fresh liver tissue (60-360 mg.) were heated to constant weight at 110°.

Experiment 28. Measurement of the amount of protein in liver tissue.

Liver tissues (50-100 mg.) were homogenised in water (2-5 ml.) and the protein content was measured using the

method of Lowry et al. [346], except that the Folin-Ciocalteu reagent was diluted with an equal quantity of water, not acid. Human serum albumin was used as a standard.

Experiment 29. Preparation of the tissue homogenate for the measurement of the enzyme activities.

The tissue was homogenised at 4° with 5mM-Tris-1mM-EDTA buffer, pH 7.5 to produce a 4-5% homogenate. This homogenate was used undiluted to determine the activity of glycogen synthetase and acid glucosidase, but samples were removed and diluted to 2% with 50mM-phosphate buffer, pH 6.5, to determine the activity of liver α-amylase. For phosphorylase and glucose-6-phosphatase the homogenate was diluted to 1% with 0.1M-citrate buffer, pH 6.4, and for phosphoglucomutase the concentration was reduced to 0.5% with 50mM-Tris-1mM-EDTA, pH 7.5.

For the determinations of the activities of the enzymes in fetal tissues higher concentrations of the homogenate were used.

The enzyme solution was kept in an ice-bath, and was only diluted to the required concentration just before the activity determination was made.

Experiment 30. Determination of glucose-6-phosphatase activity.

The activity of this enzyme is measured as the amount of phosphate released from glucose-6-phosphate in one hr. by one gram of tissue.

Reaction tubes containing 50mM-D-glucose-6-phosphate in 0.1M-sodium citrate buffer, pH 6.4 (0.1 ml.) and a 1% solution of the homogenised tissue (0.1 ml.) were mixed and incubated at 37°.[120] At 10 min. intervals the reaction was terminated in successive tubes by the addition of 1M-trichloracetic acid solution (0.2 ml.), and the amount of phosphate liberated was measured. The absorption of the blue colour was measured at 660 mμ in a UV spectrophotometer.

The determinations were repeated using 0.05 ml. of the tissue homogenate to check that the correct value for the activity had been obtained.

Incubation of 1% tissue homogenate of rat liver as described above, showed that the enzymic hydrolysis was complete after 50 min. Activity measurements were therefore carried out within 30 min.

Experiment 31. Determination of the non-specific phosphatase activity.

A 1% tissue homogenate in citrate buffer (0.1 ml.) was incubated with 50mM-β-glycerol phosphate in 0.1M-citrate

buffer, pH 6.4 (0.1 ml.) under the conditions described in the previous experiment, and the liberated phosphate was determined.

Experiment 32. Determination of phosphorylase activity.

This was determined by measuring the phosphate released from α-D-glucose-1-phosphate in the reaction:

$$G_n + \text{Glucose-1-phosphate} \rightarrow G_{n+1} + P_i$$

Tubes containing 1% solution of homogenate (0.1 and 0.05 ml.) and assay solution (0.1 ml.), which consisted of 50mM-glucose-1-phosphate, 1.5mM-adenosine monophosphate, 0.1M-sodium fluoride and 1% glycogen in 0.1M-citrate buffer pH 6.4, were incubated at 37°.(120) At 10 min. intervals the reaction was terminated by the addition of 1M-trichloracetic acid solution (0.2 ml.), the tubes being immediately removed from the incubator, and the reaction mixture diluted to 4 ml. with water. This cooling and dilution was necessary to prevent the glucose-1-phosphate in the assay solution being hydrolysed as it is acid labile. When diluted in this way the hydrolysis was found to be negligible.

The amount of phosphate released from the glucose-1-phosphate in the two sets of incubations was measured, and the activity calculated.

The reaction path of phosphorylase was investigated by carrying out incubations over a period of 90 min., and it was observed that the reaction reached completion after 60 min. confirming that 30 min. was a suitable incubation period for the determination of the activity.

Experiment 33. Determination of the activity of phosphorylase b.

The method used in the previous experiment was followed except that adenosine-monophosphate (AMP) was omitted from the enzyme assay solution.

Experiment 34. Determination of glucose-1-phosphatase activity.

This was carried out to check that the phosphate released in the preceeding experiments was cleaved from the glucose-1-phosphate by phosphorylase and not by a phosphatase in the reaction:

$$\text{Glucose-1-phosphate} \rightarrow \text{Glucose} + P_i$$

Samples of homogenate (0.1 ml.) were incubated with assay solution (0.1 ml.) containing 50mM-glucose-1-phosphate in 0.1M-citrate buffer, pH 6.4, and the amount of phosphate released was measured.

Experiment 35. Determination of the phosphoglucomutase activity.

A 0.5% tissue homogenate (0.1 and 0.05 ml.) was mixed with assay solution (0.1 ml.), which contained 8mM-glucose-1-phosphate, 8mM-magnesium chloride, 12.5mM-sodium bicarbonate and 0.1M-imidazole. To this solution was added a freshly prepared solution (0.1 ml.) of 1.25mM-cysteine in 50mM-Tris-1mM-EDTA buffer, pH 7.5, and the reaction tubes were incubated at 37°.[120]

At 5 min. intervals the reaction was terminated by the addition of 1M-trichloracetic acid solution (0.2 ml.), and the assay solutions diluted to 3 ml. with water. The solutions were boiled for 5 min. to hydrolyse all the unreacted glucose-1-phosphate, and the amount of phosphate liberated during the hydrolysis was determined. From this the activity was calculated.

To check that the glucose-6-phosphate produced by the phosphoglucomutase was not hydrolysed by 1M-trichloracetic acid solution, solutions containing 15mM, 10mM, and 5mM-glucose-6-phosphate (0.1 ml.), 1M-trichloracetic acid (0.2 ml.) and water (3 ml.) were boiled for 5 min., cooled, and the amount of free phosphate was then measured. No production of phosphate was observed, the values for phosphate present being due to the stated impurity percentage in the glucose-6-phosphate.

The reaction path of the enzyme was followed by the method described in Experiment 30, and it was found that the reaction was completed in 40-50 min. so that an incubation period of 30 min. was satisfactory.

Experiment 36. Determination of the activity of UDPG-glycogen transglucosylase.

The activity of synthetase was determined by the method of Leloir[347] by measuring the amount of UDP released from UDPG using phosphoenol pyruvate and pyruvic kinase (purchased from Boehringer).

$$UDPG + G_n \rightarrow G_{n+1} + UDP$$

$$UDP + PEP \rightarrow UTP + \text{pyruvic acid}$$

The absorption of the solution was measured in 1 cm. cells with a spectrophotometer at 520 mμ.

The reaction path of the enzyme was followed over a period of 90 min. and the enzymic action appeared to be completed after 60 min. so that the activity could be measured in 30 min.

Experiment 37. Determination of the activity of UDPG-glucosidase (a hydrolase).

As the UDP measured in the previous reaction may have been released by either glycogen synthetase or by a hydrolase, the following experiment was carried out.

Rat liver tissue (0.248 g.) was homogenised in 0.25M-glycine-8mM-EDTA buffer, pH 8.5 (5 ml.). Four sets of assay solutions were prepared as shown in the table below and the activity of the enzyme was determined in each set.

Set	Enzyme	Glycogen reagent	UDPG	Cysteine	Activity
A	0.1 ml.	0.05 ml.	0.05 ml.	0.05 ml.	41.8
B	0.1 ml.	0.05 ml.	-	0.05 ml.	1.62
C	0.1 ml.	-	0.05 ml.	0.05 ml.	3.22
D	0.1 ml.	-	-	0.05 ml.	4.02

The activity is expressed in µmoles UDP released in one hr. by one gram of tissue.

Experiment 38. Determination of α-amylase activity.

The activity of this enzyme was determined by measuring the decrease in the iodine staining of glycogen undergoing α-amylolysis.

Tubes containing 1% glycogen in 50mM-phosphate buffer, pH 6.5 (0.2 ml.) and 2% tissue homogenate (0.1 and 0.05 ml.) were incubated at 37°. At 5 min. intervals the reaction was terminated by the addition of 1M-trichloracetic acid solution (0.2 ml.). The solutions were kept at 37° for a further 10 min. to aid the coagulation and precipitation of the denatured protein. The tubes were centrifuged and the supernatant solution was decanted into tubes containing

0.2% iodine in aqueous 2% potassium iodide solution (0.2 ml.). After dilution to 5 ml. the absorption of the solutions was measured at 460 mμ in a spectrophotometer.

Experiment 39. Determination of the acid glucosidase activity.

To the assay mixture containing 20mM-maltose in 20mM-sodium acetate buffer, pH 4.5 (0.1 ml.) was added 4% tissue homogenate (0.1 and 0.05 ml.). After incubating at 37° for 10, 20 and 30 min. the reaction was terminated by heating at 100° for 3 min. The solutions were cooled and the amount of glucose produced measured using the Biochemica "Glucose Reagent".

Experiment 40. Investigation of the effect of storage on enzymes.

Tissue homogenates of different concentrations were prepared and the activities of glucose-6-phosphatase, phosphorylase, phosphoglucomutase and glycogen synthetase were determined at intervals, the enzyme solutions being stored at 4° between determinations, and being diluted to the required concentration just before analysis.

Enzyme	Concn. %	Time in hours			
		0	24	48	72
Glucose-6-phosphatase	10	495	-	488	468
	3	503	-	-	372
Phosphorylase	7	891	-	812	781
Phosphoglucomutase	3	4180	-	3744	3460
Synthetase	4	134	108	-	-

Experiment 41. Effect of premature birth on the glucose-6-phosphatase activity in rats.

Fourteen rats were delivered by Ceasarian section 18 hr. premature, and two of these were immediately killed by rapid freezing to -20°. The rest of the litter were suckled by a foster mother, and at intervals over a period of 3 days pairs of rats were removed from the litter and killed as before. The livers of the infant rats were excised and analysed for glucose-6-phosphatase activity as described in Experiment 30.

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