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Biochemical Investigation of a Case of Glycogen-Storage Disease (von Gierke's Disease)

By A. CALDERBANK,* P. W. KENT, J. LORBER, D. J. MANNERS AND A. WRIGHT
*Department of Biochemistry, University of Oxford, Department of Child Health, University of Sheffield
 and Department of Chemistry, University of Edinburgh*

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Glycogen-storage disease is characterized by the deposition of unusually large quantities of glycogen, particularly in the liver and kidneys. In a survey of a number of cases, Cori (1952-53) has recognized the existence of four types of the disease. In type 1 (also known as von Gierke's disease), the physical properties and the chemical structure of the glycogen appear to be normal and the biochemical defect lies in a striking deficiency of glucose 6-phosphatase both in the liver and kidney. Type 2 is shown by the generalized deposition of glycogen in many tissues, notably in heart and skeletal muscle. The glycogen has a normal

structure, glucose 6-phosphatase is present and the biochemical abnormality has not so far been defined. In types 3 and 4, of which few cases are known, the glycogen accumulated in the liver and kidney has an unusual structure; in type 3 it has very short outer chains and in type 4 the glycogen resembles amylopectin in having relatively long inner and outer branches.

The present studies are concerned with the investigation of a fatal case of the disease in which the molecular structure of the liver and kidney glycogen and the enzymic activity of the liver have been examined. The patient (A.K.) was a sibling of one (S.K.) investigated earlier (Manners, 1954), which corresponded to type 3 of Cori's classification. This

* Present address: I.C.I. Jeallots Hill Research Station, Bracknell, Berks.

liver glycogen (S.K.) had an average chain length of only six glucose residues (i.e. a degree of branching twice that of a normal glycogen), the molecular structure resembling that of a phosphorylase-limit dextrin. In this case, a deficiency in amylo-1:6-glucosidase was probable. Some additional properties of the S.K. glycogen are also reported.

METHODS AND MATERIALS

Normal liver. This was from a 2-year-old child who died after a short illness. The liver was removed 21 hr. after death and stored at -18° .

Diseased liver. Tissues were removed from the refrigerated body of a 4-year-old boy (A.K.) 50 hr. after death and were stored at -18° . A portion of the tissue was dropped into boiling water and heated for 10 min. Glycogen was later extracted from this boiled tissue (see Results section).

Dipotassium D-glucose 6-phosphate. This was prepared from the barium salt (104 mg.; C. F. Boehringer und Söhne, Mannheim, Germany) in water (1 ml.) by addition of solid K_2SO_4 (38 mg.). The solution was centrifuged and the supernatant fluid was made up to 2 ml. with 0.2M-potassium citrate buffer, pH 6.5.

Dipotassium α -D-glucose 1-phosphate. This was obtained by phosphorolysis of starch. A solution (0.02M) made in 6 mM-MgSO₄ was used.

Triphosphopyridine nucleotide. This was kindly given by Dr H. L. Kornberg.

Analytical methods

Glucose content. The glycogens, 0.1% concentration, were hydrolysed in 2N-H₂SO₄ at 100° for 2 hr. and the reducing sugar was determined with the Somogyi (1952) reagent.

Paper chromatography. Descending chromatograms were carried out at room temperature on Whatman no. 1 paper with ethyl acetate-pyridine-water (10:4:3, by vol.) as solvent, and aniline oxalate as development reagent.

Absorption spectra of iodine complexes. The conditions of Peat, Whelan, Hobson & Thomas (1954) were used.

Periodate oxidation. The glycogens were oxidized at room temperature with potassium metaperiodate (Bell & Manners, 1952). The liver glycogen was also oxidized with a limited excess of sodium metaperiodate under the conditions of Manners & Archibald (1957).

Glycogen value. A solution of concanavalin A was prepared from a 0.9% NaCl extract of jack-bean meal (Cifonelli & Smith, 1955). For the determination of glycogen values, 9 ml. of the above solution was added to 1 ml. of aqueous glycogen solution containing 0.1–1.0 mg. of polysaccharide, and the extinction measured after 10 min. at 420 m μ with a Unicam SP. 500 spectrophotometer. The extinction given by 1 mg. of glycogen under these conditions was compared with that of a standard sample of rabbit-liver glycogen (glycogen value 1.00) kindly provided by Professor F. Smith (see Cifonelli, Montgomery & Smith, 1956).

Viscosity measurements. The viscosity of glycogen solutions of various concentrations was measured at 25° in a modified Ubbelohde viscometer. The limiting viscosity number $[\eta]$ was obtained by linear extrapolation of η sp./C to zero concentration (C as g./ml.).

Phosphate estimations. These were performed by the method of Berenblum & Chain (1938).

Enzymic degradation: α - and β -amylolysis. The enzyme preparations and conditions of hydrolysis were those described by Liddle & Manners (1957). α -Amylolysis results are given as P_M values, i.e. apparent percentage conversion into maltose.

Isoamylase. This enzyme (cf. Manners & Khin Maung, 1955) was extracted from brewer's yeast by Miss Z. H. Gunja.

Enzymic assays

Glucose 6-phosphatase estimation. Homogenates were prepared by grinding 1 g. of abnormal liver in a chilled mortar. The homogenate was stirred with ice-cold glass-distilled water, filtered through muslin and the filtrate made up to 3.5 ml. A homogenate of normal liver (800 mg.) was prepared similarly except that the final volume of the supernatant fluid was 1.8 ml. Incubations were carried out at 30° for 15, 30 or 60 min., when the reaction was terminated by the addition of 1 ml. of trichloroacetic acid (10%, w/v). Water (1 ml.) was then added and the mixture was centrifuged (445 g for 5 min.). A sample (1 ml.) of the supernatant liquid was withdrawn for determination of phosphate.

Phosphoglucomutase estimation. Abnormal liver (1 g.) was homogenized with glass-distilled water (3 ml.) in a Potter glass homogenizer. The product was centrifuged (30 min. at 10 000 g) and the supernatant liquid was used for enzyme assay immediately, and after standing for 24 hr. at 2° and being diluted 10-fold, 100-fold and 500-fold with the cysteine buffer. Glucose 1-phosphate was incubated at 30° with the enzyme preparation (0.1 ml.), in the presence of 0.05M-cysteine brought to pH 7.5 by addition of 0.1N-NaOH, for 5 or 10 min. The reaction was terminated by the addition of 5N-H₂SO₄ (1 ml.) and the solution was diluted with water to a final volume of 5 ml. The resulting solution was heated on a boiling-water bath for 3 min., cooled, centrifuged (445 g, 3 min.) and 1.5 ml. of the supernatant was withdrawn for determination of phosphate.

Glucose 6-phosphate dehydrogenase estimation. Abnormal liver (1 g.) was homogenized with water (4.5 ml.) in an all-glass apparatus at 0° and centrifuged (3500 rev./min.) for 20 min. The supernatant liquid was used for enzyme assay. In parallel experiments, normal liver (850 mg.) was homogenized in water (2.6 ml.) in the same way. Incubation mixtures were prepared containing 0.25M-glycylglycine (pH 7.6, 3.5 ml.); 0.1M-MgCl₂ (3.5 ml.); water (5.6 ml.); enzyme preparation (0.7 ml.); triphosphopyridine nucleotide (TPN; 3.5 ml. containing 1.4 mg.). For the 'zero time' reading (i.e. immediately after addition of the enzyme), 2.4 ml. of the mixture was mixed with ethanol (2.4 ml.) and 10% (w/v) Na₂SO₄ (0.1 ml.). After 15 min. the solution was centrifuged (2000 rev./min.) and the extinction of the supernatant was measured at 340 m μ . The reaction commenced when 0.05M-dipotassium glucose 6-phosphate (0.6 ml.) was added to the remaining incubation mixture at 20°. Samples (2.4 ml.) were withdrawn and investigated at various intervals in the manner described. Spectral measurements were made in comparison with an 'enzyme blank' identical with 'zero time' incubation mixture save that the TPN was substituted by an equivalent volume of water.

RESULTS

Isolation and purification of A.K. glycogen

The boiled liver tissue (243 g.) was ground with sand and extracted three times with boiling water in an atmosphere of nitrogen. The combined extracts were cooled to 0° and deproteinized by the addition of 0.1 vol. of 40% (w/v) trichloroacetic acid. After removal of protein, the glycogen was precipitated with ethanol, purified by five further precipitations and dried with ether. Yield, 18.9 g. (equivalent to 7.8% yield from the tissue).

Glycogen was similarly extracted from boiled kidney tissue (78 g.). Yield 3.4 g. (i.e. 4.4% yield from the tissue). Extraction of the kidney residue with 30% (w/v) KOH at 100° did not yield any additional glycogen.

Boiled brain and muscle tissue were extracted successively with water and 30% KOH at 100°. No glycogen could be isolated.

Characterization of purified A.K. glycogens

The liver glycogen had $[\alpha]_D +196^\circ$ (c, 0.2 in water), and on acid hydrolysis gave glucose (96%) and no other sugar. The aqueous solution stained deep red-brown with iodine ($\lambda_{\max}$ 460–465 m μ ; $E_{\max}$ 0.21).

The kidney glycogen had the following properties: $[\alpha]_D +200^\circ$ (c, 0.2 in water); glucose content 98%; iodine complex, $\lambda_{\max}$ 445–450 m μ , $E_{\max}$ 0.19.

Periodate oxidation. Liver glycogen (262.2 mg.) gave 5.06, 5.11 and 5.11 mg. of formic acid after potassium metaperiodate oxidation for 9, 14 and 16 days. The final figure corresponds to an average chain length of 14.6 glucose residues. In a duplicate experiment, 261.0 mg. of liver glycogen gave 5.17 mg. of formic acid on complete oxidation, equivalent to average chain length 14.5.

Kidney glycogen (253.7 mg.) was similarly oxidized; the final production of formic acid, after 17 days, was 5.06 mg., equivalent to average chain length 14.3. In a repeat oxidation, 253.7 mg. of glycogen gave 5.07 mg. of formic acid, i.e. average chain length 14.1.

Liver glycogen (100.3 mg.) in water (22 ml.) was then oxidized at 2° with 3 ml. of 8% (w/v) sodium metaperiodate. The final production of formic acid, after 20 days' oxidation, was 2.0 mg., corresponding to an average chain length 14.4 glucose residues.

Glycogen values. The measurement of the extinction of 1 mg. of A.K. glycogen in the presence of concanavalin-A gave glycogen values: liver glycogen, 0.97; kidney glycogen, 1.01. Samples of amylopectin isolated from potato starch (var. Kerr's Pink) and the starch from the freshwater alga *Dunaliella bioculata* (Eddy, Fleming & Manners, 1958) did not react with concanavalin-A.

Limiting viscosity number. In 0.1 M-NaCl solution, the liver and kidney A.K. glycogens had $[\eta]$ 8.3 and 5.3 respectively. These figures are about one-twentieth of those for amylopectin.

Enzymic degradation

α -Amylolysis. The results of a comparison of the action at 37° of purified salivary α -amylase on the human glycogens, and on control samples of rabbit-liver glycogen (average chain length 13.8) and potato amylopectin (average chain length 22.0) are shown in Table 1.

β -Amylolysis. On treatment with barley β -amylase at pH 4.6 and 37°, the liver and kidney glycogens both had a β -amylolysis limit of 46%. Under similar conditions, the rabbit-liver glycogen and potato amylopectin gave 53 and 61% conversion into maltose.

β -Amylolysis after pretreatment with isoamylase. The glycogens (26–28 mg.) were incubated with isoamylase (50 mg.) at pH 5.9 for 24 hr. at 20° in a total volume of 10 ml. The enzyme was inactivated by heating; 5 ml. of 0.2 M-acetate buffer, pH 4.6, β -amylase (2600 units) and water to 25 ml. were then added. After 24 hr. at 37° the β -amylolysis limits were: liver glycogen 60%, kidney glycogen 59%. Potato amylopectin on similar treatment had a β -amylolysis limit of 77%. The outermost branch points in these polysaccharides are therefore of the α -1:6-glucosidic type.

Further investigation of S.K. glycogen

This glycogen, the structure of which has already been discussed by Manners (1954), gave a pale-yellow-brown stain with iodine and the solution did not show an absorption peak in the region 440–480 m μ ; at 430 m μ the extinction was only 0.05. The glycogen had glycogen value 1.61, $[\eta]$ 10.6 in 0.1 M-NaCl and, on α -amylolysis (see Table 1), the P_M values were 24, 27 and 33 respectively. After treatment with isoamylase, the β -amylolysis limit was 20%.

Table 1. *Action of salivary α -amylase on polysaccharides*

The polysaccharides (0.5 mg./ml.) were incubated at 37° with freeze-dried α -amylase (2.3 units/ml.) in the presence of 0.05% NaCl in a total volume of 100 ml. Samples (5 ml.) were analysed, at intervals, for reducing sugar (as maltose).

Time of incubation (hr.) ...	P_M		
	2.5	5	24
Liver glycogen (A.K.)	62	66	74
Kidney glycogen (A.K.)	63	67	76
Rabbit-liver glycogen	65	69	75
Potato amylopectin	74	79	89

Determination of liver-enzyme activities

A survey of the activity of four enzymes, namely glucose 6-phosphatase, phosphoglucumutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase, was carried out on homogenates of the diseased (A.K.) liver. Results of six experi-

ments showed that with incubation times between 15 and 30 min. the tissue hydrolysed glucose 6-phosphate at a rate of 1.81–2.72 μ moles/hr./g. of frozen tissue. Under identical conditions, enzyme preparations of normal liver hydrolysed the substrate much more quickly, at a rate of 129.8–130.0 μ moles/hr./g. of frozen tissue.

Table 2. *A comparison of the properties of glycogens from glycogen-storage disease*

Property	Kidney glycogen (A.K.)	Liver glycogen (A.K.)	Liver glycogen (S.K.)
$[\alpha]_D$ (water)	+200°	+196°	+201°
Iodine complex λ_{max} . (m μ)	445–450	460–465	—
Glycogen value	1.01	0.97	1.61
Average chain length (glucose residues)	14.2	14.5	6.1
α -Amylolysis limit (%)	76	74	33
β -Amylolysis limit (%)	(b)* 46	46	14
	(a)* 59	60	20
Molecular weight (see Addendum)	7×10^6	7×10^6	2×10^6
Limiting viscosity number	5	8	11

* (b) Before and (a) after treatment with isoamylase.

Table 3. *Glucose 6-phosphatase activity of abnormal- and normal-liver homogenate*

In these experiments the substrate was 0.1M-dipotassium glucose 6-phosphate (0.1 ml.) in 0.1M-potassium citrate buffer (pH 6.5). The enzyme preparations are as described in the Methods and Materials section. Final volume, 0.5 ml. Results of duplicate experiments are shown in parentheses.

Additions	Duration of incubation (min.)	Phosphate found (μ g.)	Phosphate liberated/hr./g. of frozen tissue (μ moles)
Abnormal liver (A.K.)			
Buffer (0.2 ml.); enzyme (0.2); water (0.1 ml.)	60	32.5	—
Buffer (0.2 ml.); substrate; water (0.2 ml.)	60	3.75	—
Buffer (0.2 ml.); substrate; enzyme (0.2 ml.)	0*	37.2	—
Buffer (0.2 ml.); substrate; enzyme (0.2 ml.)	15	38.0 (38.4)	1.81 (2.72)
Buffer (0.2 ml.); substrate; enzyme (0.2 ml.)	30	39.5 (39.5)	2.60 (2.60)
Buffer (0.2 ml.); substrate; enzyme (0.2 ml.)	60	42.0 (42.0)	2.72 (2.72)
Normal liver			
Buffer (0.3 ml.); enzyme (0.1 ml.); water (0.1 ml.)	15	40.0	—
Buffer (0.3 ml.); substrate; water (0.1 ml.)	15	12.25	—
Buffer (0.3 ml.); substrate; enzyme (0.1 ml.)	15	97.0	129.8
Buffer (0.3 ml.); enzyme (0.1 ml.); water (0.1 ml.)	30	38.9	—
Buffer (0.3 ml.); substrate; water (0.1 ml.)	30	13.7	—
Buffer (0.3 ml.); substrate; enzyme (0.1 ml.)	30	148.8	130.0

* Zero-time control; trichloroacetic acid was added before the enzyme.

Table 4. *Phosphoglucumutase activity of abnormal liver*

The reaction mixtures were made with 0.02M-dipotassium α -glucose 1-phosphate (0.1 ml.) as substrate. The buffer mixture contained 0.05M-cysteine (0.2 ml., pH 7.5) and 6 mM-MgSO₄ (0.1 ml.). The enzyme preparation is as described in the Methods and Materials section. Final volume, 0.4 ml.; incubated at 30°. Results of duplicate experiments are shown in parentheses.

Additions	Duration of incubation (min.)	Phosphate liberated (μ g.)	Hydrolysable phosphate used (μ moles)
Enzyme; buffer	10	26.65	—
Enzyme; buffer; substrate*	10	93.0	—
Substrate; buffer; water (0.1 ml.)	10	67.7	—
Enzyme; substrate; buffer	5	31.3 (29.3)	2.04 (2.10)
Enzyme; substrate; buffer	10	31.3 (30.0)	2.04 (2.08)

* Zero-time control; sulphuric acid was added before the enzyme.

Table 5. *Phosphoglucomutase activity of aged and diluted enzyme preparations of abnormal liver*

Tubes contained 0.05M-cysteine (0.2 ml.) and enzyme (0.1 ml., appropriate preparations are described in the Methods and Materials section). In some cases, 6 mM-MgSO₄ (0.1 ml.) was also added. Final volume, 0.4 ml.; incubated at 30° for 5 min.

Additions	Hydrolysable P remaining (μg.)	Glucose 1-phosphate used (μmoles/hr./g. of frozen tissue)
Enzyme (1:10); MgSO ₄	7.37	—
Enzyme (1:100); MgSO ₄	4.37	—
Glucose 1-phosphate; water (0.1 ml.)	68.2	—
Glucose 1-phosphate; enzyme (1:10)	40.7	5088
Glucose 1-phosphate; enzyme (1:100)	63.0	1536
Glucose 1-phosphate; enzyme (1:500)	66.0	1704

Table 6. *Glucose 6-phosphate dehydrogenase activity of abnormal and normal liver*

The enzyme preparation and incubation mixture is described in the Methods and Materials section; 0.24 μmole of TPN was present.

Time (min.)	TPN reduced (μmoles/g. of frozen tissue)	
	Abnormal liver	Normal liver
0	—	—
2	2.32	0.49
5	3.06	2.78
10	5.36	6.30
15	7.37	6.42
20	8.85	7.73

On the other hand, the diseased tissue was found to possess a high level of phosphoglucomutase activity. The enzyme activity was measured by the method of Najjar (1948), in which an enzyme preparation is incubated with dipotassium α-glucose 1-phosphate and the residual glucose 1-phosphate is estimated by acidic hydrolysis and determination of the liberated inorganic phosphate.

Table 4 shows the rapid action on glucose 1-phosphate in 10 min. at 30°. A further experiment (Table 5), in which the enzyme preparation was diluted (1:10), showed that the substrate is used at a rate of 5088 μmoles/hr./g. of frozen tissue. At higher dilutions (1:100; 1:500), the rate fell to 1536 and 1704 μmoles/hr./g. of frozen tissue respectively [Weber & Cantero (1957) give values for 37°].

Because of the turbidity of homogenates of the diseased liver, a modification of the method of Glock & McLean (1953, 1956) was used for determination of the glucose 6-phosphate dehydrogenase activity of the tissue. In this modification, the enzymic reaction was terminated by the addition of ethanol, and addition of Na₂SO₄ facilitated flocculation of the opalescent substance. The rate of reduction of TPN by the diseased liver was 3.06 μmoles/g. of frozen tissue in 5 min., in contrast with a value of 2.78 μmoles/g. of frozen normal tissue.

A qualitative investigation of the phosphohexoisomerase activities of normal- and diseased-liver homogenates by the method of Glock & McLean (1956) showed that both tissues were highly active.

Glycogen content of liver and other tissues

The content of glycogen in the abnormal muscle and liver was determined by the method of Kemp & Kits van Heijningen (1954) (performed by Dr A. J. M. van Beusekom-Kits van Heijningen). No fixed glycogen was to be found in the muscle, and, in the liver, the amount of glycogen extracted by cold trichloroacetic acid varied between 9.91 and 10.50 %, and a further 0.53–0.67 % of non-extractable polysaccharide remained. The amount of free sugars (including glucose 1-phosphate) was 0.06–0.09 % (Table 7).

The nitrogen content (Kjeldahl) of homogenates of the diseased liver was 21–23 mg. of nitrogen/g. of frozen tissue, whereas for the normal liver the values were 28.6–29.2 mg. of nitrogen/g. of tissue.

DISCUSSION

The present case of glycogen-storage disease is characterized by the accumulation of liver and kidney glycogen which appears to have a 'normal' structure. Glycogen is not deposited in the skeletal muscles, i.e. glycogenesis is not generalized, as in the type 2 disease. Although samples of human liver and kidney glycogen from comparable normal tissues were not available for study, the branching properties of the A.K. glycogens are similar to those of the majority of mammalian glycogens hitherto examined (for a review of glycogen structure, see Manners, 1957).

The interaction of the A.K. glycogens with iodine and concanavalin-A, the average chain length determined by periodate oxidation, the α-amylolysis limit and the low limiting viscosity number differentiate them from amylopectins, and hence eliminate the possibility of a deficiency in amylo-1:4 → 1:6-transglucosidase (branching

Table 7. *Glycogen content of diseased liver (A.K.)*

The pairs of figures represent the results of duplicate experiments.

	Free glycogen* (%)	Fixed glycogen (%)	Total (%)	Free sugars† (%)	
				Anthrone	H ₂ SO ₄ reaction
Immediately deproteinized	10.21, 10.50	0.53, 0.63	10.74, 11.03	0.09, 0.06	0.06, 0.06
Stored for 9 hr. at room temp.	9.91, 10.38	0.60, 0.67	10.58, 10.95	0.09, 0.09	0.09, 0.09

* Values for cold trichloroacetic acid extract corrected for free sugar values.

† 80% (v/v) methanol extracts.

enzyme) as the biochemical lesion (type 4 disease). The extent of β -amylolysis shows that the outer chains contain, on the average, about nine glucose residues and are clearly much longer than those in phosphorylase-limit dextrins. The above reactions are consistent with the view that the present case corresponds to type 1 disease, i.e. a true von Gierke's disease. It follows that the probable enzymic deficiency is of glucose 6-phosphatase and experimental investigations have shown that this is in fact the case.

The previous conclusions (Manners, 1954) on the unusually high degree of branching in the S.K. glycogen have been confirmed. The significantly greater reaction with concanavalin-A, and lower P_M value on α -amylolysis are to be expected; the extremely weak iodine-binding power from measurements of absorption spectra is also of interest. The S.K. and A.K. liver glycogens thus show very marked differences in molecular structure (see Table 2). It is unlikely that these glycogens were markedly changed during their isolation, particular precautions (exclusion of oxygen, etc.) being taken to minimize degradation.

The 'normal' structure of the A.K. glycogen suggests that the biochemical lesion does not reside in a deficiency of amylo-1:6-glucosidase, as has been found by Illingworth & Cori (1952) and Illingworth, Cori & Cori (1956) for some forms of glycogen-storage disease characterized by glycogen accumulation in the heart and kidneys as well as in the liver.

A survey of the activities of glucose 6-phosphatase, phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase in the diseased liver revealed that the first of these was present in much smaller amounts than in normal human liver from a child of similar age. The other enzymes were highly active. An enzyme preparation from the diseased liver (A.K.) resulted in the liberation of 8.3 μ g. of inorganic phosphate/hr./100 mg. of liver from glucose 6-phosphate, compared with 406 μ g. with normal liver. Despite unavoidable differences in the age of the diseased and normal tissue, these figures clearly indicate a marked difference in the levels of glucose 6-phosphatase activity. In two cases of the disease re-

ported by Cori & Schulman (1954), values were 20 and 17 μ g. of inorganic phosphate liberated/hr. as compared with 362 μ g. for normal liver.

The present case thus corresponds to type 1 of Cori's classification, i.e. a case of von Gierke's disease in which the biochemical lesion is a much reduced level of glucose 6-phosphatase. In these circumstances glucose 6-phosphate may be expected to be retained in the liver, not being available to other tissues without removal of the phosphate group. Its conversion into glucose 1-phosphate and hence into glycogen is thus favoured either by the action of the usual phosphorylase mechanisms or by the alternative route suggested by the results of Leloir & Cardini (1957).

The high concentration of free glycogen (10.21; 10.50%) in the frozen diseased liver is characteristic of the disease. The slight decrease in the values (9.91 and 10.38%) after the liver has been kept at room temperature for 9 hr. suggest that little glycogenolysis has occurred during storage of the liver. It has to be said, however, that the diseased liver was not obtained until 50 hr. after the death of the patient and, although the body was refrigerated, the possibility of some changes in glycogen content during this period cannot be excluded. Furthermore, the diseased liver was stored at -18° for 3 months before it was examined. It has not been possible to obtain 'normal tissue' of comparable age and origin.

It is of interest that the present case (A.K.; boy, 4 years old) of type 1 glycogen-storage disease is a sibling (brother) of the patient (S.K.; girl, 12 years old) who suffered from the type 3 disease (Manners, 1954).

Two other cases of siblings having glycogen-storage disease have been reported (Illingworth & Cori, 1952; Cori & Cori, 1952). The first was a girl (M.A.S.) aged 15 months having type 1 von Gierke's disease; a sibling died with similar symptoms though biochemical details were not given. The second was a case of male twins (Daniel B. and Dennis B., aged 3½ years). In both, the structures of the liver glycogens were apparently 'normal', and examination of the liver of Dennis B. showed a decreased level of glucose 6-phosphatase activity. These twins may be presumed to have suffered from

the type 1 form of the disease. Another such family has also been reported by di Sant'Agnese, Andersen, Mason & Bauman (1950).

SUMMARY

1. Liver glycogen ($[\alpha]_D + 196^\circ$ in water) isolated in a case of glycogen-storage disease has a normal structure (average chain length 14.5) and resembles rabbit-liver glycogen in respect of α - and β -amylolysis and the action of isoamylase.

2. The diseased liver has high phosphoglucomutase, glucose 6-phosphate dehydrogenase and phosphohexoisomerase activities.

3. Glucose 6-phosphatase is present in the diseased liver in much reduced quantity, indicating that the disease corresponds to type 1 of Cori's classification, i.e. von Gierke's disease.

4. The present case (A.K.) is a sibling of one (S.K.) investigated earlier and found to be suffering from type 3 of the disease, i.e. a deficiency of amylo-1:6-glucosidase. It is notable that these siblings suffered from different forms of glycogen-storage disease.

5. The kidney glycogen (A.K.) was also examined and was found to have a normal structure.

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ADDENDUM

Examination in the Ultracentrifuge

By A. G. OGSTON

Department of Biochemistry, University of Oxford

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Samples of kidney and liver glycogen (A.K.) were examined in a Svedberg oil-turbine ultracentrifuge at 350 rev./sec. and at about 21° ; the bar-schlieren optical system (Baldwin, 1953) was used. To allow direct comparison with the previously reported values of Bell, Gutfreund, Cecil & Ogston (1948), the glycogen concentration was 1 g./100 ml. in m-sodium chloride.

The forms of the boundaries were similar to those observed by Bell *et al.* (1948), both materials being highly polydisperse, the liver glycogen rather more so than the kidney glycogen; in addition, the liver glycogen contained a small amount of material sedimenting as a distinguishable boundary

two to three times as fast as the main boundary. Calculated from the maximum ordinates of the main boundary peaks (Cecil & Ogston, 1948) and corrected to the values for water at 20° , the sedimentation coefficients (at 1 g./100 ml.) were: kidney glycogen 106s; liver glycogen 102s. A value of 0.65 was assumed for the partial specific volume (Bell *et al.* 1948).

DISCUSSION

The sedimentation coefficients of these samples were higher than any of those found by Bell *et al.* (1948) for various samples of glycogen similarly

Fig. 1. O, Sedimentation coefficients plotted against molecular weight for samples of glycogen investigated by Bell *et al.* (1948). The full curve was drawn freehand and used to estimate the approximate molecular weights of the present samples of liver glycogen (●) and kidney glycogen (■).

prepared and measured under the same conditions. The approximate average molecular weights of the latter samples (determined by sedimentation and

diffusion, at 1 g./100 ml.) are plotted in Fig. 1 against the sedimentation coefficients. Extrapolation of this curve indicates, for the present samples, average molecular weights of approximately 7.1×10^6 (kidney glycogen) and 6.6×10^6 (liver glycogen).

Orrell & Bueding (1958) have reported much higher values for sedimentation coefficients of glycogens, and Stetten, Katzen & Stetten (1958) have found much higher values of molecular weights by light-scattering. It is not clear how far these differences may be due to the use of different methods of extraction or to measurement under different conditions.

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The Preparation and Purification of Cytidine 2':3'-Phosphate

By E. M. CROOK, A. P. MATHIAS AND B. R. RABIN

Department of Biochemistry, University College London, Gower Street, London, W.C. 1

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In the degradation of ribonucleic acid by pancreatic ribonuclease, uridine and cytidine 2':3'-phosphates are formed as intermediates and subsequently hydrolysed to the corresponding 3'-phosphates (Markham & Smith, 1952; Brown, Dekker & Todd, 1952). The kinetics of the action of ribonuclease on ribonucleic acid are complex (Vandendriessche, 1953) and interpretation awaits detailed investigations with substrates of low molecular weight. A first step in the logical approach to this problem must involve studies of the hydrolysis of the cyclic substrates. Thus it is necessary that these should be available in reasonable quantity and a high degree of purity. Both chemical and enzymic methods are available for the preparation of these substances. The chemical methods are to be preferred since the isolation of the product is difficult when the enzymic method is employed. The chemical methods involve intramolecular esterification of the adjacent hydroxyl group of the nucleoside 2'- or 3'-phosphate; both trifluoroacetic anhydride (Brown, Magrath & Todd, 1952) and dicyclohexyl carbodi-imide (Dekker & Khorana, 1954) have been used as condensing agents. The

trifluoroacetic anhydride method appeared to be the easier route and we have investigated it in some detail.

EXPERIMENTAL AND RESULTS

Cytidylic acid. This (a mixture of cytidine 2'- and 3'-phosphates) was prepared by the method of Cohn & Carter (1950), De-Acidite FF (formate form) being used in place of Amberlite IRA 400.

Trifluoroacetic acid. This was obtained from Eastman Kodak Co., Kirby Industrial Estate, Liverpool.

Trifluoroacetic anhydride. This was prepared by the method of Swarts (1922), with the modification that the proportion of trifluoroacetic acid to phosphorus pentoxide was 40:65 (w/w).

Ribonuclease. This (lot no. 21630) was obtained from Armour and Co. Ltd., Hampden Park, Eastbourne, Sussex.

Paper chromatography. Whatman no. 4 paper, previously washed with 2 N-acetic acid, then with water by descending chromatography, was employed. The chromatograms were developed by the ascending method for 14 hr., with propan-2-ol-water-aq. NH_3 soln. (sp.gr. 0.88) (70:25:5, by vol.) as solvent (Brown, Magrath & Todd, 1952). The papers were dried in a stream of air and the nucleotides detected by examination in u.v. light. In some instances the