GLYCOGEN ACCUMULATION IN THE LIVER DUE TO 2 : 4–DINITROPHENOL.

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SINCE Loomis and Lipmann (1948) discovered that 2 : 4-dinitrophenol (DNP) uncouples oxidation and phosphorylation many investigations have been performed *in vitro* to find the precise mechanism of this effect. However the cellular changes induced by a prolonged administration of DNP *in vivo* have not been examined recently. Older work concerning the toxic properties of this drug and of related compounds has been fully reviewed by several authors (Magne, Mayer and Plantefol, 1932 ; Tainter and Cutting, 1933 ; Heymans and Casier, 1935 ; Staub, 1935).

Recently there has been put forward the hypothesis that the uncoupling of oxidation and phosphorylation could play an important rôle in the pathogenesis of the cellular metamorphoses (Ciaranfi, 1953), and at least as regards the cloudy swelling, such a hypothesis is supported by several experimental results (Fonnesu, 1952, 1954 ; Fonnesu and Severi, 1953, 1954*a*). Thus, experiments have been undertaken to ascertain whether a prolonged DNP-intoxication produced in cells of living animals a morphological alteration, which might be fitted into any of the pictures of classical cell pathology. It has been observed, in the early stages of the intoxication, that both liver and kidney were affected by cloudy swelling while, on the other hand, the other organs examined revealed rather moderate and inconstant lesions. Prolonged treatment with DNP produced in the kidney, in addition to cloudy swelling, the appearance of necrotic foci, while in the liver a quite unexpected change occurred resembling that seen in glycogen storage disease (Fonnesu and Severi, 1954*b*). This last observation appeared of notable interest and deserved further and more extended investigation.

It has recently been observed that liver glycogen is increased in rats poisoned with dinitro-*o*-cresol (DNOC) (Parker, Barnes and Denz, 1951). Immediately after the injection of the drug, liver glycogen decreases, but after a time it tends to increase above normal (Barnes, 1953). According to Barnes there are three possible mechanisms to explain the glycogen accumulation by DNOC : (*a*) delayed absorption of food from the intestine, with the imposition of what amounts to a fast on the animal ; (*b*) action of DNOC on the adrenals, and (*c*) conversion into glycogen by the liver of metabolites which could accumulate during the acute phase of poisoning. The first mechanism, however, appeared improbable, since in fasted rats to which glucose was given orally followed by DNOC the same amount of glycogen was synthesised as in controls receiving glucose alone. Evidence which could decide in favour of one of the other proposed mechanisms has not been obtained. In the experiments with DNOC it has proved impossible to ascertain whether the adrenals play a rôle in bringing about the changes in liver glycogen

levels after DNOC. On the other hand, no experimental result has been found supporting the third hypothesis.

The present paper is concerned with a study, both histological and biochemical, on the accumulation of glycogen in the liver following administration of DNP.

EXPERIMENTAL.

Experimental animals.

Adult Wistar rats (strain WAG-2A) of either sex were used throughout these experiments. The rats were given subcutaneously, twice a day, approximately 10 hr. apart, a saturated aqueous solution of DNP. Each injection was equivalent to 22 mg. DNP/kg. body wt. and the treatment was prolonged for 20 days. Since, at the beginning, the animals were more sensitive to DNP, a slightly lower dose was administered during the first three days (15 mg. DNP/kg. body wt. for each injection). The DNP saturated solution (0·56 per cent) was prepared by dissolving the compound in dilute NaOH, after which the solution was adjusted to pH 7 with dilute HCl. Normal rats of the same stock were used as controls. Care was taken to use for each treated animal a control of the same sex corresponding, as far as possible, in age and weight.

The treated animals were used 15 hr. after the last DNP injection. At this time the rats were less lively than the normal ones on account of the chronic poisoning, but they did not show any signs which could be accounted for by an acute effect of DNP.

Both normal and experimental rats were given *ad libitum* a mixed diet up to 12 hr. prior to the experiment, at which time food was withdrawn. Non-fasting rats were only used when liver glycogen was extracted for the determination of iodine absorption spectra.

The rats were stunned by a blow on the back of the head and then decapitated; pieces of liver, kidney and skeletal muscle were removed as quickly as possible for histological examination and biochemical investigations. In some cases glycogen determinations were also carried out in liver samples kept at room temperature or at 37° for 24 hr. after death.

Experiments (In Vitro).

Enzyme studies in liver.—As soon as removed, the hepatic tissue was homogenised in 2 vol. of ice-cold water. Homogenates were then diluted according to the enzymic activity which had to be assayed. The activities of acid and alkaline phosphatases, of glucose-6-phosphatase and of phosphoglucomutase were measured by the procedure of Cori and Cori (1952).

The reaction systems were incubated in a Warburg apparatus for 1 hr. at 30°. After this period, 1 vol. of 10 per cent trichloroacetic acid was added and inorganic phosphorus was determined in 1 ml. of filtrate. Each experiment included, in addition to the complete systems, samples incubated with water instead of the substrate and others containing the substrate but water in place of the homogenate.

The composition per ml. of the complete reaction mixtures was as follows:

Acid phosphatase: 0·3 ml. of 0·1 M acetate buffer, pH 5; 0·5 ml. of 0·03 M sodium β-glycerophosphate (British Drug Houses Ltd., U.K.), pH 5; 0·2 ml. of homogenate (equivalent to 4 mg. of fresh liver).

Alkaline phosphatase: 0·3 ml. of 0·1 M veronal buffer, pH 8·6; 0·5. ml. of 0·03 M sodium β-glycerophosphate, pH 8·6; 0·2 ml. of homogenate (equivalent to 20 mg. of fresh liver).

Glucose-6-phosphatase: 0·3 ml. of 0·1 M citrate buffer, pH 6·8; 0·5 ml. of 0·01 M potassium glucose-6-phosphate (prepared from the barium salt obtained from Schwarz Laboratories, Inc., U.S.A.), pH 6·8; 0·2 ml. of homogenate (equivalent to 2 mg. of fresh liver).

Phosphoglucomutase: The composition of the reaction mixture was the same as that for glucose-6-phosphatase, but potassium glucose-1-phosphate (Schwarz Laboratories, Inc., U.S.A.) was substituted for potassium glucose-6-phosphate and the substrate solution also contained 0·003 M cysteine (Eastman Kodak Co., U.S.A.) and 0·01 M-$MgCl_2$ to ensure the maximal activity of the mutase. In these experiments easily hydrolysable phosphorus was determined, before and after incubation, in addition to the inorganic phosphorus. As glucose-1-phosphate is totally hydrolysed in 7 min. in 1 N-HCl at 100° and as glucose-6-phosphate is very resistant to acid hydrolysis, the values obtained enabled us to calculate the amount of glucose-1-phosphate converted to glucose-6-phosphate, and how much of the latter remained unchanged and how much was hydrolysed to glucose and inorganic phosphorus.

Isolation and purification of liver glycogen.—Isolation and purification of liver glycogen were performed following essentially the method of Somogyi (1934) with slight modifications which, though causing a loss of some glycogen, made it possible to obtain highly purified glycogen samples. Material for the extractions consisted of several livers obtained respectively from groups of normal and poisoned rats.

Tissues were minced and then digested with 30 per cent NaOH (2 vol. per wt. of tissue) in a boiling water bath for 3 hr. On cooling a layer of soap appeared on the surface of the liquid, from which it was possible to separate the underlying clear fluid. The soapy layer was discarded as proposed by Illingworth, Larner and Cori (1952). From the clear alkaline fluid glycogen was precipitated by adding ethanol until a final alcohol concentration of 33 per cent was reached. After several hours at room temperature, the precipitate was separated by centrifugation, washed repeatedly with a mixture of 1 vol. of ethanol and 2 of 20 per cent NaOH (until the washings appeared colourless) and finally re-washed with ethanol. This impure glycogen was re-dissolved in distilled water and the solution adjusted to pH 4–5 with HCl. Ethanol was added up to a final alcohol concn. of 33 per cent (at which, in an acid medium, glycogen does not precipitate); a brownish precipitate appeared in a few hours, which was separated by centrifugation and discarded. The ethanol concn. in the clear supernatant liquid was then brought to 45 per cent to precipitate the glycogen. This was separated by centrifugation and repeatedly washed with 45 per cent ethanol followed by 95 per cent ethanol and by ether. The ether was allowed to evaporate and the glycogen dried *in vacuo* to constant weight. The purity of the compound was checked by determining the glucose equivalents of a glycogen solution before and after hydrolysis in N-HCl for 4 hr. at 100°. The physical characteristics of isolated glycogen samples were those as described by Somogyi (1934).

Iodine absorption spectrum of liver glycogen.—The iodine absorption spectra of the isolated liver glycogens were determined according to Illingworth and Cori (1952), using a Beckman spectrophotometer mod. DU.

Histological methods.—Sections were stained with haematoxylin and eosin

and with Best's ammoniacal carmine for glycogen. Other sections were stained with iodine solution, before and after incubation with saliva.

Analytical methods.—Glycogen was determined by a modification (Stadie, Haugaard and Marsh, 1951) of the method of Good, Kramer and Somogyi (1933), using the method of Nelson (1944) for the final determination of glucose equivalents.

Phosphorus was determined according to Fiske and SubbaRow (1925).

A Coleman spectrophotometer mod. S.14 was used.

Experiments In Vivo.

Preliminary experiments consisted of blood glucose determinations both in normal and poisoned animals kept without food for 12 hr. Experiments were then performed to investigate the effects of several glycogenolytic agents on the stored glycogen and to study the glucose tolerance. In these experiments the rats had access to food up to 2 hr. prior to the tests. Blood samples were taken by heart puncture before and at various intervals after the injection.

Blood glucose was determined in duplicate according to Nelson's photometric adaptation of Somogyi's method (Nelson, 1944).

Epinephrine.—Epinephrine hydrochloride 1/1000 was injected subcutaneously in doses of 0·08 mg./100 g. body wt.

Hyperglycaemic-glycogenolytic-factor (HGF).—HGF (Eli Lilly and Co., U.S.A., batch No. 208–108B–148A) was used, 2 mg. being dissolved in 2 ml. 0·08 N-NaOH; the solution was incubated at 39° for 2 hr. and the pH adjusted to 7·4 with dil. HCl. The solution was then diluted to a final vol. of 10 ml. with distilled water and was injected intracardially in doses equivalent to 10 μg. of " crude " HGF/100 g. body wt.

Anterior pituitary somatotropic hormone (STH).—A purified preparation of STH (" Antuitrin Growth," Parke, Davis & Co.) was used. The hyperglycaemic activity of the STH solution was tested in preliminary experiments, since the activity of this preparation decreases progressively with time. Both normal and treated rats received intraperitoneally the smallest dose capable of causing a rapid and transient hyperglycaemia.

Glucose.—The glucose tolerance was determined after intracardiac injection of a 40 per cent glucose solution in doses of 100 mg./100 g. body wt.

Statistical analysis.—All the data were analysed statistically. Symbols and formulae are those given by Fisher (1950).

RESULTS.

Histological Findings.

Liver sections stained with haematoxylin and eosin showed that prolonged poisoning with DNP produced a characteristic change. At low magnifications, trabeculae appeared enlarged and close to one another; the lobular pattern of the liver was lost. At high magnification, parenchymal cells, with well-defined cell membranes, appeared blown up and tightly packed together. Nuclei were enlarged and slightly paler than normal and the cytoplasm appeared empty (Fig. 2). The degree of change, however, varied from one area to another of the same section.

The histological changes induced by DNP in kidney and skeletal muscle were those observed previously (Fonnesu and Severi, 1954*b*),

Sections stained with Best's ammoniacal carmine showed that normal liver cells contained only a few granules of glycogen, as one would expect, since the livers were obtained from fasting animals (Fig. 3). In contrast, liver cells of DNP-poisoned rats appeared filled with glycogen, although these rats had also fasted for 12 hr. prior to the experiments (Fig. 4). The appearance of sections stained with iodine solution, before and after incubation with saliva, confirmed these results.

Sections of other organs from poisoned animals showed no difference from the normal when stained for glycogen.

Biochemical Investigations.

In Table I the results of glycogen determinations in the tissues of the control and DNP-treated rats are shown. The data are referred to wet wt., since the water content of DNP livers has been found unchanged from the normal (Fonnesu and Severi, 1954*b*).

The glycogen content of the livers from DNP-treated animals was much higher (about 7-fold) than that of the normal rats. On the other hand, the glycogen content of kidney and skeletal muscle from the treated animals was not significantly changed.

TABLE I.—*Glycogen Content of Tissues of DNP-treated Rats Compared with Controls.*

mg. glycogen/100 mg. fresh tissue. Animals fasted for 12 hours.

	Liver.		Muscle.		Kidney.	
	Controls.	DNP-treated rats.	Controls.	DNP-treated rats.	Controls.	DNP-treated rats.
1	0·25	3·10	0·50	0·45	0·06	0·09
2	0·35	1·28	0·35	0·45	0·09	0·05
3	0·10	1·77	0·50	0·32	0·08	0·05
4	0·70	1·60	0·25	0·30	0·07	0·07
5	0·42	2·45	0·62	0·45	0·05	0·05
6	0·55	0·95	0·45	0·35	0·09	0·08
7	0·45	1·90	0·30	0·45	0·04	0·06
8	0·30	0·95	0·42	0·65	0·07	0·07
9	0·25	2·15	0·50	0·50	0·05	0·07
10	0·25	2·35	0·45	0·65	0·06	0·07
11	0·32	1·92	0·52	0·35	—	—
12	0·25	1·35	0·65	0·55	—	—
13	0·40	1·40	0·60	0·60	—	—
14	0·10	1·15	0·35	—	—	—
15	0·25	2·60	0·30	—	—	—
16	0·30	1·65	0·40	—	—	—
17	0·15	1·75	0·40	—	—	—
18	0·10	4·10	—	—	—	—
19	—	2·40	—	—	—	—
20	—	2·30	—	—	—	—
Means ± S. E. M.	0·30± 0·037	1·95± 0·171	0·44± 0·028	0·46± 0·033	0·06± 0·006	0·06± 0·005
Difference between the means	1·65		0·02		—	
t	8·70		0·46		—	
P	<0·01*		>0·05†		—	

* Highly significant. † Not significant.

In both normal and pathological livers, glycogen totally disappeared when tissues were kept for 24 hr. at room temperature or at 37°.

The results of the enzymic studies in liver are summarised in Table II.

TABLE II.—*Enzymes in Livers of Normal and DNP-treated Rats.*

Mean values of 8 observations: 100 mg. fresh liver, 60 min., 30°.

Enzyme.	Acid phosphatase (P liberated from β-glycerophosphate at pH 5).		Alkaline phosphatase. (P liberated from β-glycerophosphate at pH 8·6).		Glucose-6-phosphatase. (P liberated from glucose-6-phosphate at pH 6·8).		Phosphoglucomutase. (P of glucose-6-phosphate formed from glucose-1-phosphate at pH 6·8).			
							Remained unchanged.		Hydrolysed to inorg. P.	
Normal and treated rats.	Normal.	Treated.	Normal.	Treated.	Normal.	Treated.	Normal.	Treated.	Normal.	Treated.
Mean (μg.)	619	395	33	8	1680	1720	1773	1780	143	122
S.E.M.	85	56	3	0·8	155	143	167	209	69	34
Difference between the means	224		25		20		7		21	
t	2·39		8·5		0·19		0·03		0·27†	
P	<0·05*		<0·01†		>0·05‡		>0·05‡		>0·05‡	

* Significant. † Highly Significant. ‡ Not significant.

The data presented show that both acid and alkaline phosphatase activities were significantly decreased in liver from DNP-poisoned rats. On the other hand, the enzyme directly involved in glycogen degradation in liver, namely glucose-6-phosphatase, appeared unchanged. In the experiments with glucose-1-phosphate as substrate, in which maximal activity of phosphoglucomutase was ensured by the addition of cysteine and $MgCl_2$, the same amount of glucose-1-phosphate was converted into glucose-6-phosphate by both normal and pathological livers. The quantity of the latter hydrolysed to glucose and inorganic phosphorus was the same when the systems contained either normal or pathological liver homogenates. Consequently no difference appeared in the amount of glucose-6-phosphate, which was found unchanged at the end of incubation.

The iodine absorption spectra of the glycogen isolated from livers of normal and poisoned rats were practically identical and appeared equally chromogenic with an absorption peak at 470 mμ. The data concerning the glycogen of normal rat liver correspond to those found by Illingworth and Cori (1952) for glycogen of normal human liver.

Table III states the blood glucose level in normal and DNP-treated rats fasted for 12 hr. In DNP rats the level is significantly higher than in the controls.

The results concerning the effects of the glycogenolytic agents and the glucose load are presented in the form of curves showing average changes in blood glucose concentration. It will be observed that the blood glucose remained practically constant during the control period preceding the injections.

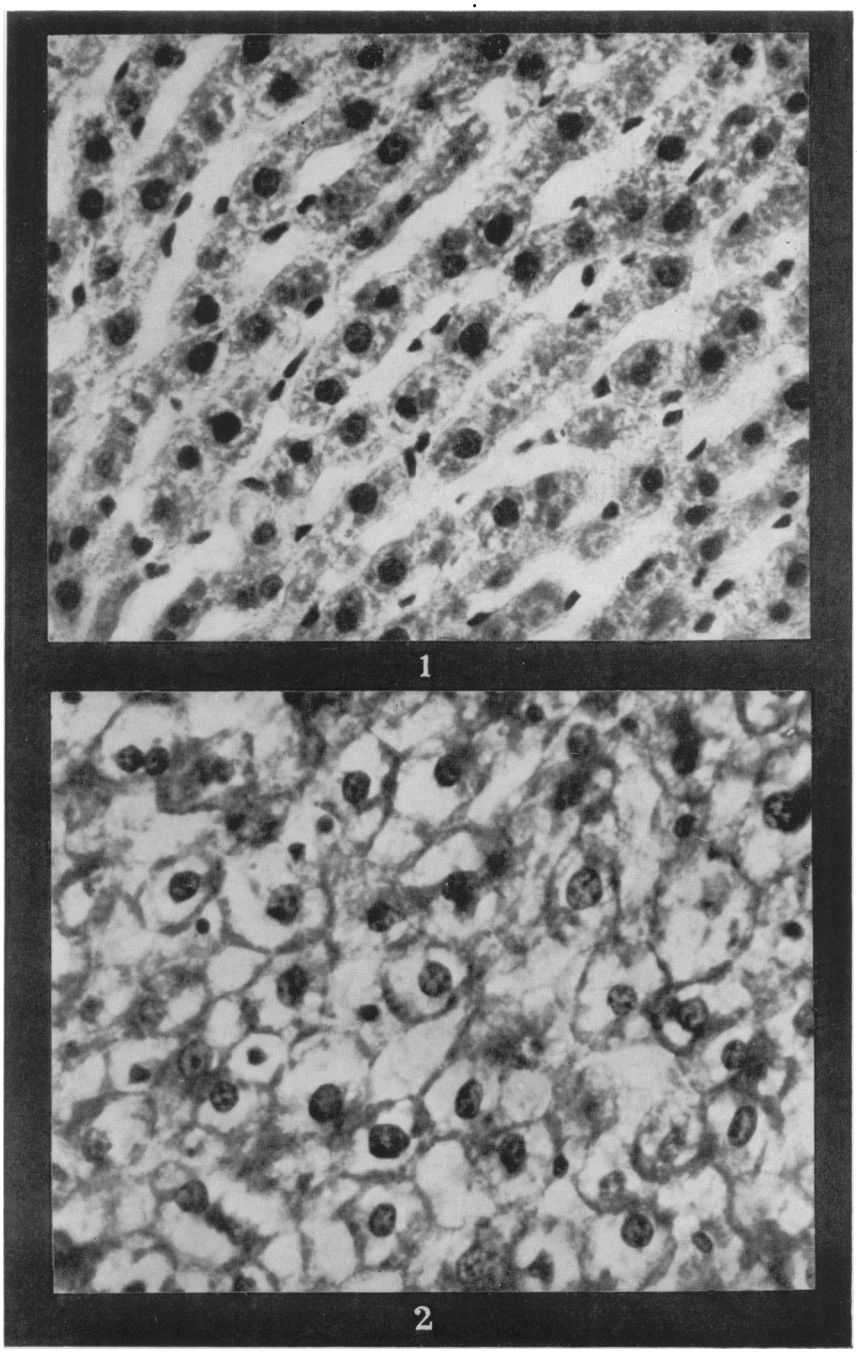
EXPLANATION OF PLATES.

FIG. 1.—Liver of normal rat fasted for 12 hours. Haematoxylin and eosin. (× 560).

FIG. 2.—Liver of DNP-treated rat fasted for 12 hours. Haematoxylin and eosin. (× 560).

FIG. 3.—Liver of normal rat fasted for 12 hours. Best's ammoniacal carmine for glycogen. (× 560).

FIG. 4.—Liver of DNP-treated rat fasted for 12 hours. Best's ammoniacal carmine for glycogen. (× 560).



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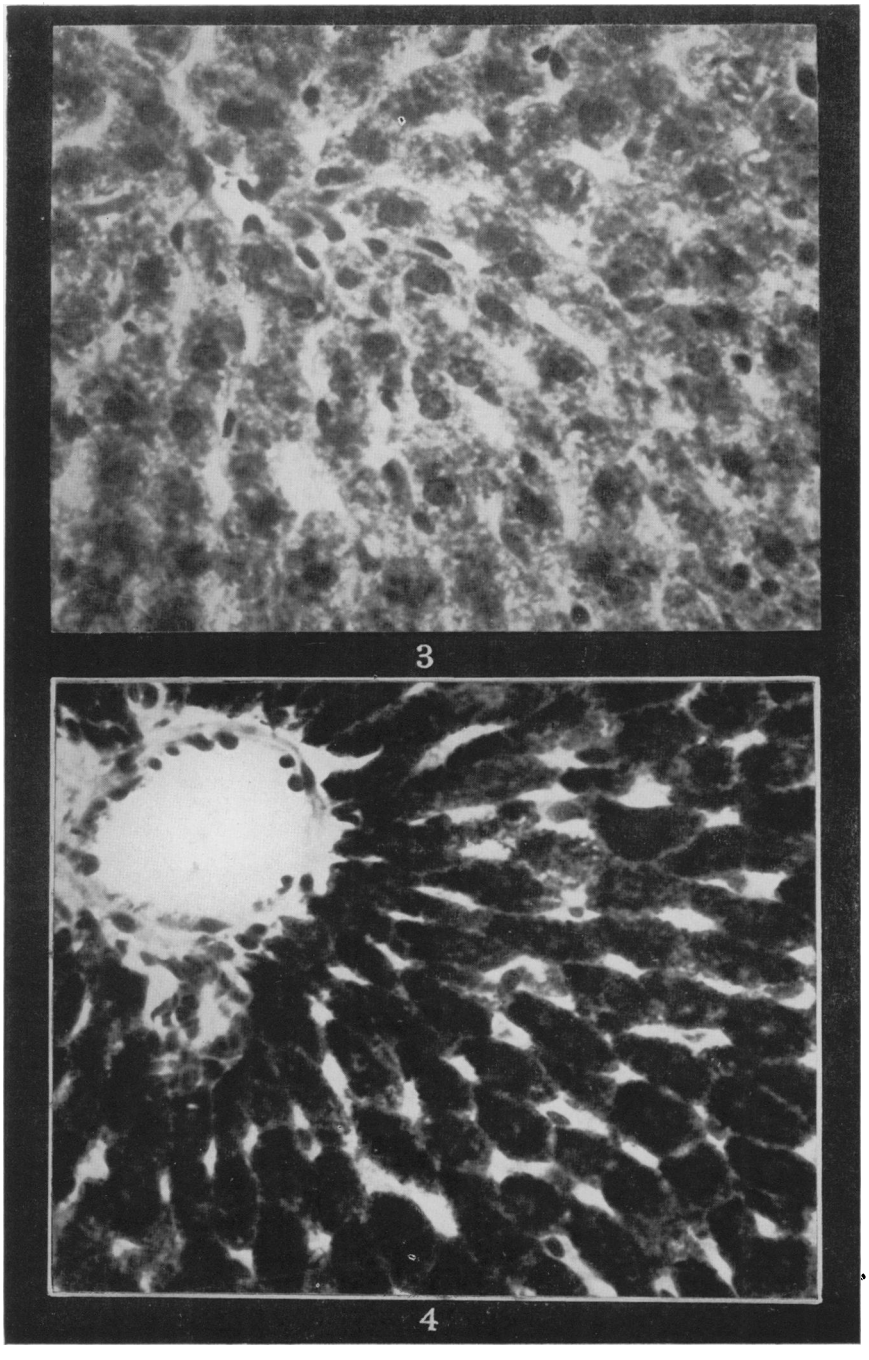

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TABLE III.—*Blood Glucose Concentration in Normal and DNP-treated Rats Fasted for* 12 *Hours* (*mg.*/100 *ml.*).

	Normal.	DNP-treated.
1	78	90
2	76	114
3	74	100
4	88	108
5	86	100
6	96	102
7	82	108
8	70	112
9	82	104
10	86	132
11	88	103
Means ± S. E. M.	82±2·26	106±3·21
Difference between the means	24	
t	6·1	
P	<0·01*	

* Highly significant.

Fig. 5 represents experiments in which rats received a subcutaneous injection of epinephrine hydrochloride. Following the injection the blood glucose rose in both control and test animals, but the hyperglycaemic response was much greater in the latter. In normal rats the peak rise was reached in about 2 hr. and was followed by a rapid decrease. In the treated rats the glucose rise was much greater, and the maximum was reached after 5 hr.

The experiments in which animals were given HGF intracardially gave results shown in Fig. 6. The injection of HGF was followed, in both normal and DNP-poisoned rats, by a prompt and marked hyperglycaemia, but the increase was more pronounced and prolonged in DNP-treated rats than in controls. Taking into account that large doses of HGF were used to obtain the maximal change, our data for normal animals appear well in agreement with the results of others (Foá, Santamaria, Berger, Smith, and Weinstein, 1952 ; Kirtley, Waife and Peck, 1953 ; Ingle, Nezamis and Humphrey, 1953).

The hyperglycaemic response produced by STH in normal and treated rats (Fig. 7) was similar to, although much smaller than that produced by epinephrine or HGF.

Fig. 8 shows that the glucose tolerance was markedly reduced in DNP-poisoned rats as compared with normal controls.

DISCUSSION.

Evidence has been presented (Fig. 1–4 ; Table I) that in rats treated repeatedly with DNP, a glycogen accumulation occurs in the liver in the later stages of the treatment. Such a change is strictly limited to the liver, since in the other organs the glycogen levels appear unaltered from the normal. It has already been mentioned that analogous results have been obtained by poisoning rats with DNOC (Parker *et al*, 1951 ; Barnes, 1953), a compound similar to DNP. These observations undoubtedly are unexpected because it is known that liver glycogen decreases after a single injection of DNP or DNOC.

The change seen in the liver following prolonged treatment with DNP resembles a picture characteristic in human pathology, known as von Gierke's or glycogen storage disease (for literature see van Creveld, 1939). In von Gierke's disease, however, glycogen storage, which may be enormous, is generally not limited to the liver, but occurs also in other organs such as the kidney, heart, muscles and brain ; large amounts of fat are also present in the tissues. The deposits of both

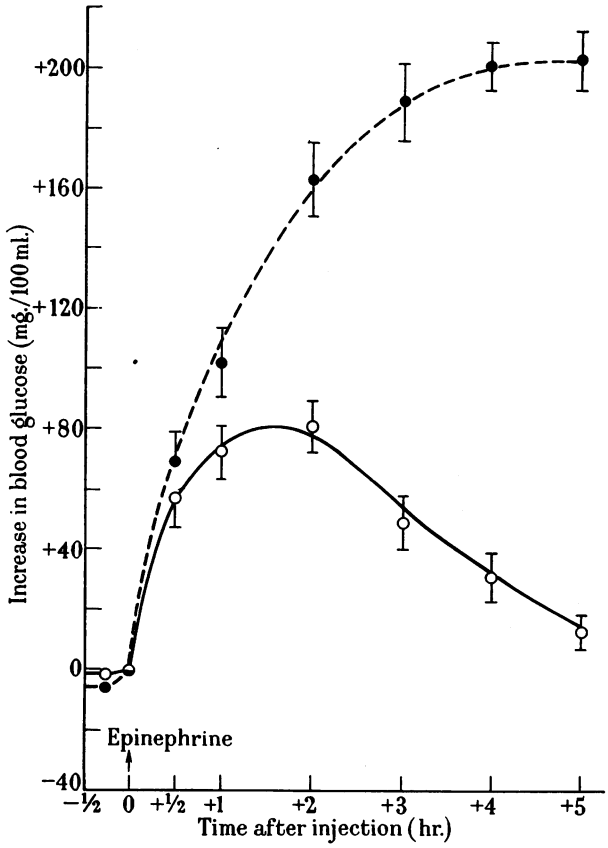

FIG. 5.—Blood glucose changes in normal (○———○) and DNP-treated rats (●- - - - - -●) following an epinephrine injection. Each point represents the mean of six animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

glycogen and fat cause a marked enlargement of the organs, with the exception of the spleen, the hepatomegaly without splenomegaly being characteristic for the condition. In patients with von Gierke's disease, the liver glycogen is not readily available for blood glucose formation as shown by the tendency to hypoglycaemia, by the flat blood glucose curve after epinephrine injection and by the persistence of the glycogen in tissues for an unlimited time after death. In contrast, in DNP-poisoned rats the glycogen accumulates only in the liver and disappears totally after death. As compared with normal controls, the DNP

rats exhibit a greater blood glucose concentration (see Table III) and a higher blood sugar curve after epinephrine injection (see Fig. 5). On the other hand, the fat content in DNP livers is lower than normal (Fonnesu and Severi, 1954*b*), this being a further difference from von Gierke's disease.

It should be noted here that the levels of liver glycogen found in fasted DNP animals (Table I) can occur in well fed normal rats, though they are not found in

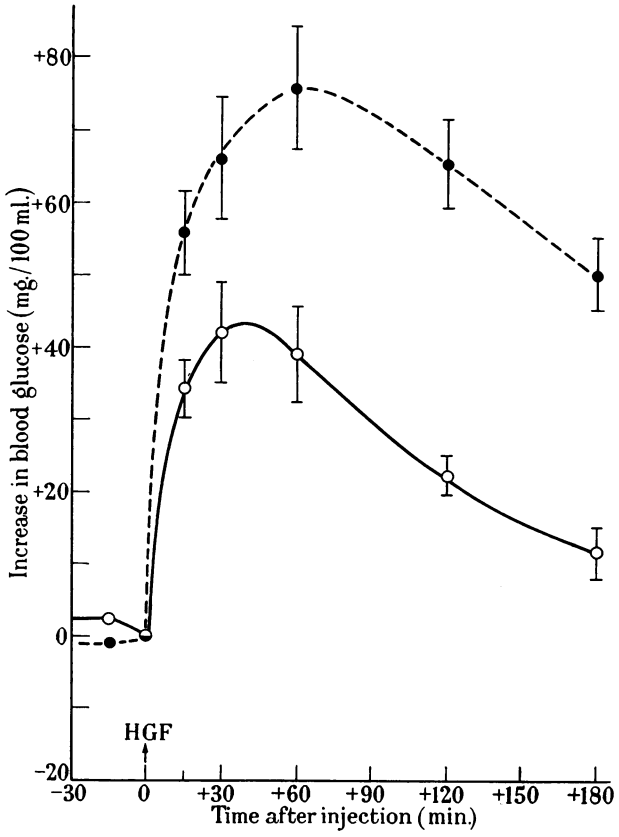

FIG. 6.—Blood glucose changes in normal (○———○) and DNP-treated rats (●- - - - -●) following a HGF injection. Each point represents the mean of six animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

normal fasted rats. Therefore, the enlargement of the cells in DNP livers (Fig. 2) cannot be attributed exclusively to the glycogen accumulation. Since cloudy swelling occurs in liver in the early stages of DNP-poisoning (Fonnesu and Severi, 1954*b*), it is reasonable to assume that glycogen accumulates in swollen cells. This view is supported by the finding that nuclei are also markedly enlarged (Fig. 2) as compared with the normal (Fig. 1).

The following considerations led us to investigate the glucose-6-phosphatase activity in livers from normal and DNP-poisoned animals. The conversion of glycogen to glucose in the liver requires several enzymes, *viz.*, phosphorylase, amylo-1 : 6-glucosidase (Cori and Larner, 1950), phosphoglucomutase and glucose-

6-phosphatase. For glycogen synthesis from glucose hexokinase and the branching enzyme are required in addition to phosphorylase and phosphoglucomutase. Thus two enzymes, amylo-1 : 6-glucosidase and glucose-6-phosphatase, bring about glycogen degradation without intervening in its synthesis. A reduction of these enzymes could cause glycogen storage in the liver, since a deficiency of enzymes also involved in glycogen synthesis would have been incompatible with the accumulation of the polysaccharide in liver. However, the post-mortem glycogen disappearance

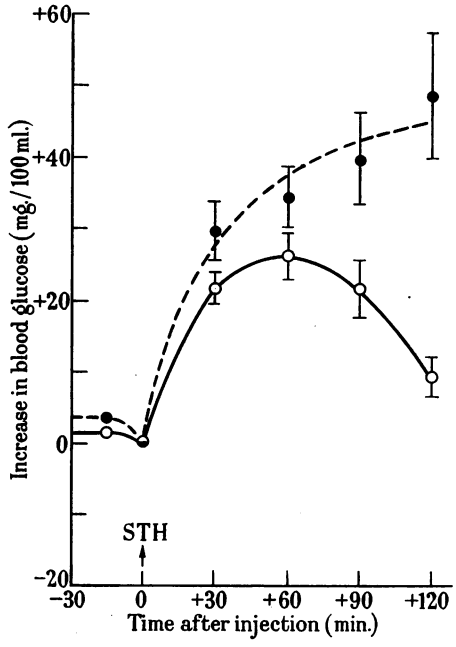

FIG. 7.—Blood glucose changes in normal (○———○) and DNP-treated rats (●- - - - -●) following a STH injection. Each point represents the mean of nine animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

evidently excluded, in our case, a defect of amylo-1 : 6-glucosidase, by whose action the complete splitting of the glycogen molecule by phosphorylase is conditioned (Cori and Larner, 1951). Since the glucose-6-phosphatase activity has been measured and found unchanged (Table II), it is possible to conclude that the glycogen liver storage by DNP does not depend on a deficiency of the enzymes involved in glycogen degradation. As can be seen from the data summarised in Table II, the decrease of acid and alkaline phosphatases is the only enzymic change observed in DNP livers. This enzymic deficiency, however, cannot account for the glycogen accumulation in liver and must be regarded as a concomitant disturbance.

The glycogen accumulation in DNP treatment could not be attributed to an abnormality in the structure of glycogen such that the enzymes were unable to attack the polysaccharide molecule. In fact, apart from the post-mortem glycogen disappearance in DNP livers, there is evidence that carbohydrate stores in poisoned animals are well mobilised by glycogenolytic agents (see Fig. 5–7). However,

this does not exclude the possibility that some structural alterations, compatible with the enzymic degradation, might exist in the glycogen molecule. Therefore it appeared of interest to ascertain whether or not the liver glycogen formed after DNP poisoning exhibited a normal iodine absorption spectrum. It is known that the absorption spectrum of polysaccharides in the presence of iodine may give information about the structure of the polysaccharide. However, the iodine absorption spectrum of liver glycogen from poisoned animals is normal.

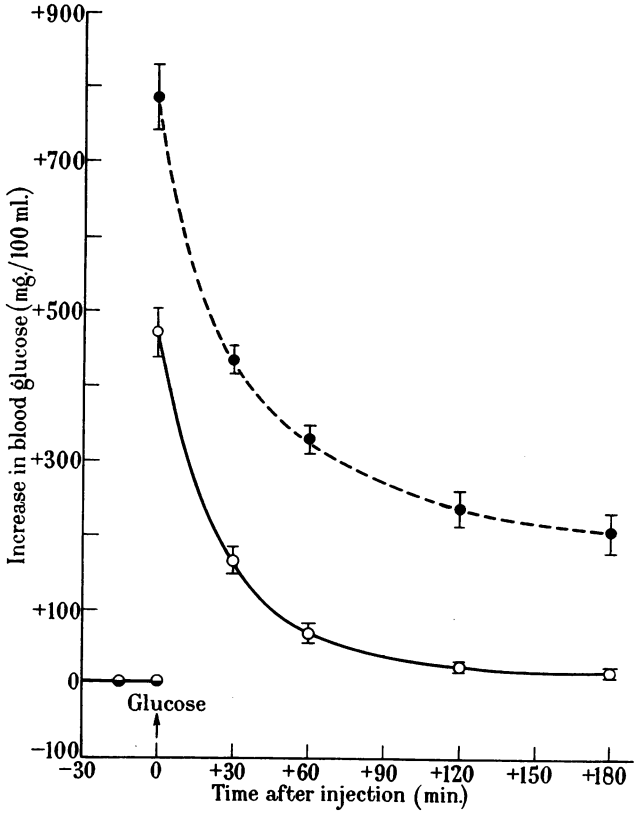

FIG. 8.—Glucose tolerance curve in normal (○———○) and DNP-treated rats (●- - - - -●). Each point represents the mean of eight animals. The vertical lines represent the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

The changes in blood glucose induced by epinephrine, HGF and STH, both in normal and intoxicated rats, merit some discussion. It has been long known that epinephrine and HGF produce hyperglycaemia in normal animals by causing increased hepatic glycogenolysis. Sutherland and Cori (1951) have shown that they increase the concentration of active phosphorylase in liver, this effect being sufficient to explain their hyperglycaemic action. According to these authors, the phosphorylase content of the liver would not be static, but would represent a balance between an active and an inactive form of the enzyme. The rapid change in either direction would be brought about by an enzyme system in the liver upon which epinephrine and HGF would act.

It appears evident that the blood glucose rise after the injection of glycogenolytic agents depends on an adequate supply of liver glycogen. When the glycogen stores are depleted as in fasting (Weisberg, Caren, Huddlestun and Levine, 1949 ; Pincus, 1950) and uncontrolled experimental diabetes (Pincus, 1950), the effects of glycogenolytic agents do not fully manifest themselves.

These considerations led us to investigate the effects of epinephrine and HGF on the liver glycogen of poisoned rats. Both epinephrine and HGF produced in DNP-treated animals a greater and more prolonged hyperglycaemia than in controls (Fig. 5 and 6).

One might suppose that the glycogen liver accumulation was due to a deficiency of HGF. Experiments with HGF did not prove this, since they showed only that the HGF injected was active on liver glycogen. However, the above supposition might be easily controlled by investigating the effects of a factor stimulating HGF secretion. For this purpose STH was used because this hormone, in addition to reducing insulin secretion (Anderson and Long, 1947) and inhibiting peripheral utilisation of glucose (Milman, DeMoor and Lukens, 1951), produces hyperglycaemia by stimulating the alpha cells of the pancreas to secrete HGF (Foá, Magid, Glassman and Weinstein, 1953). A comparison of Fig. 6 and 7 shows that the blood glucose changes after STH were very similar to those after HGF. The smaller hyperglycaemic response after STH can be explained by the fact that the amount of HGF secreted under STH stimulation was certainly much smaller than that of HGF injected directly.

The results obtained with epinephrine, HGF and STH concordantly show that in rats treated with DNP the blood glucose rise is more pronounced than in normal controls after the injection of glycogenolytic agents. This fact certainly depends on the increased glycogen content of the liver of intoxicated rats. It seemed possible, however, that at least in part it may be due to a reduced glucose utilisation by the tissues because of biochemical alterations induced by DNP.

It appeared, therefore, of interest to investigate the glucose tolerance, which was seen to be markedly reduced in DNP-treated animals (Fig. 4). The decreased rate of disappearance of the injected glucose from the blood cannot be attributed to the raising of renal threshold. It has been observed that the threshold for glucose is lowered in the kidney in cloudy swelling (Agostini, 1953) and that the kidneys of DNP-treated rats show parenchymatous degeneration (Fonnesu and Severi, 1954*b*).

It seems reasonable to conclude that the reduced glucose tolerance is due to a decreased utilisation of the injected glucose. This conclusion is also supported by the higher blood glucose level in fasting DNP-treated rats.

With this in mind, it is possible to attempt the interpretation of the glycogen accumulation by DNP. The reduced glucose utilisation in peripheral tissues caused by DNP would lead to the raising of blood glucose level, which would induce the glycogen accumulation in liver through the known homoiostatic mechanism (Soskin and Levine, 1946 ; Frank, 1949). The equilibrium between formed glycogen and split glycogen would thus be altered in favour of the former. It is evident, however, that in this case it is necessary to concede that the DNP liver must have a normal or only slightly decreased capacity of synthesising glycogen. Thus, the alteration leading to glycogen accumulation will not lie in the liver but in the decreased glucose utilisation in the peripheral tissues which will produce hyperglycaemia.

The proposed mechanism recalls the effects of the adrenocortical hormones in the carbohydrate metabolism, *viz.*, (*a*) increased gluconeogenesis, (*b*) marked increase of liver glycogen with slight modification of the glycogen muscle content, and (*c*) reduced peripheral utilisation of carbohydrates. Whether the results obtained are attributable to a direct action by DNP or to an indirect action mediated by the adrenals remains to be established.

SUMMARY.

It has been shown by histological and chemical methods that on prolonged treatment of rats with DNP a glycogen accumulation takes place in the liver.

Liver glycogen disappeared totally after the death of the animal.

The blood glucose level in fasting DNP-treated rats was raised.

Glucose-6-phosphatase and phosphoglucomutase were unchanged in the liver of DNP-poisoned rats, whilst both acid and alkaline phosphatases were decreased.

The iodine absorption spectrum of glycogen isolated from livers of DNP rats was unmodified as compared with the spectrum of glycogen from normal rat liver.

The effects of epinephrine and HGF on liver glycogen have been investigated. Both these glycogenolytic agents produced in DNP-treated animals a greater and more prolonged hyperglycaemia than in normal controls. Analogous results were obtained by stimulating the secretion of HGF with STH.

The glucose tolerance was markedly reduced in DNP-treated rats.

A hypothesis explaining the action of DNP in causing glycogen accumulation is proposed.

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While this paper was in preparation, a paper by Smith (1954) was published showing that the effect of sodium salicylate on liver glycogen and blood glucose of rats was similar to that of DNP. A state of eucorticalism, rather than a stimulation of the adrenal cortex, is sufficient for the effect of salicylate on liver glycogen and blood glucose to be demonstrated. It is probable that the mechanisms involved in the salicylate effect are the same as those of DNP and DNOC.

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