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The Effect of Dinitro-*o*-cresol on the Deposition of Liver Glycogen in the Rat

By J. M. BARNES

Medical Research Council Unit for Research in Toxicology, Serum Institute, Woodmansterne Road, Carshalton, Surrey

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During the course of some experimental observations on the toxicity of dinitro-*o*-cresol (DNOC) a histological examination was made of the tissues of rats that had received a series of daily injections of a toxic dose (20 mg./kg.). The histological examination was essentially negative except for a somewhat unusual appearance of the liver cells. Special staining showed the liver to be packed with glycogen (Parker, Barnes & Denz, 1951). This was an unexpected observation because it is generally believed that the immediate effect of an injection of DNOC is to deplete the liver of its glycogen.

The effect of single and repeated doses of DNOC on the liver glycogen was, therefore, examined. The experiments showed that while the immediate effect of DNOC is to deplete the liver glycogen, the process is later reversed. The 24 hr. fasting levels of liver glycogen are always higher than normal in rats treated with one or a series of daily injections of DNOC.

In the course of the work an attempt was made to reduce the individual variation in the liver glycogen of normal fasting rats by submitting the animals to regular feeding regimes.

The normal practice in the animal house was to place in the cage a supply of rat cubes that was more than adequate for the needs of the rat during the next 24 hr. If food is suddenly withdrawn from these animals for a 24 hr. fast, the animals may differ in the amount of food each will have in its stomach at the time the food is withdrawn and the actual fast of some rats might be longer than 24 hr.

A regular feeding regime of two feeds a day, each of 1 hr. duration, and a single feed of 1 hr. was instituted. There was no reduction in the range of variation in individual animals, but the general fasting levels differed significantly.

These observations are discussed.

METHODS

Determination of liver glycogen, blood sugar and lactate. The liver glycogen was determined by the method of Good, Kramer & Somogyi (1933) on pieces of liver removed as quickly as possible from rats killed by guillotine. Although care was taken not to excite the animals immediately before death, an experiment was done that suggested that such a precaution was probably unnecessary. Two groups of four normal well-fed rats were killed. One group was kept quiet and killed, and the rats in the other group were excited just before being caught and killed. The glycogen levels in the two groups were 6.92 ± 2.9 and $7.2 \pm 0.72\%$ respectively. Blood sugar was determined by the method of Somogyi (1945) and blood lactate by that of Barker & Summerson (1941). All the results are expressed as means, together with the standard error.

*Dinitro-*o*-cresol injections.* DNOC purified by recrystallization from 50% ethanol was injected subcutaneously as a 1% (w/v) aqueous solution adjusted to pH 7. The dose was usually 20 mg./kg. which produces severe symptoms of poisoning. In order to reduce the number of deaths when the animals were to be given a series of injections, the cages were placed in a cool draughty corridor immediately after the animals had been injected.

Feeding regimes

Albino rats weighing 150–250 g. were used.

'Continuous feeding.' The rats were given each day a supply of cubes (Medical Research Council diet 41) more than adequate for their needs so that they had continuous access to food during the 24 hr.

Two meal regime. The rats were given cubes from 9–10 a.m. and 2–3 p.m. each day and had access to water at all times.

Single meal regime. The rats were given cubes only from 9 to 10 a.m. each day.

In the experiments with DNOC the rats were either on 'continuous feeding' or trained to eat a single feed for 1 hr. each day. In the latter case all rats were placed on the single feed regime for at least 3 weeks before they were used for experiments with DNOC to allow them to become accustomed to this new feeding regime.

RESULTS

Effect of feeding regime on fasting liver glycogen

The liver glycogen after a 24 hr. fast falls, but the extent of the fall depends upon the type of feeding regime to which the animals had been accustomed before being fasted.

Table 1. *The effect of the previous feeding regime on the liver glycogen of rats fasted for 24 and 48 hr.*

(a: rats with access to food throughout the 24 hr. b: rats accustomed to access to food for 1 hr. each day. Results expressed as means with standard errors.)

Feeding regime	Duration of fast (hr.)	No. of rats	Liver glycogen (%)
a	24	47	0.085 $\pm$ 0.015
	48	6	0.26 $\pm$ 0.07
b	24	53	0.70 $\pm$ 0.07
	48	12	0.31 $\pm$ 0.09

The results of a number of different observations have been collected and are presented in Table 1. They show that rats trained to eat their food during 1 hr. each day have a higher fasting level of liver glycogen than those accustomed to the continuous feeding regime. If the fast is continued for 48 hr. the liver glycogen levels of rats accustomed to continuous feeding rises again. In the rats trained to hourly feeds the level continues to fall, but not to levels as low as those seen in the continuously fed rats fasted for only 24 hr.

When rats were suddenly placed on the regular feeding regimes they took some time to adjust themselves to the new conditions and at first they lost weight, but by the end of 3 weeks they had regained this and were growing again.

Table 2. *Effect of different feeding regimes on the liver glycogen levels in normal fasting rats*

(All rats killed after 24 hr. fast. Each group contained 4 rats. Each group killed after 1, 2 and 3 weeks on the different feeding regimes. These regimes were (a) access to food for 1 hr. each 24 hr.; (b) access to food for two periods of 1 hr. each 24 hr.; and (c) access to food throughout the 24 hr. Results expressed as means with standard errors.)

Feeding regime	Liver glycogen (%)		
	1st week	2nd week	3rd week
a	1.04 $\pm$ 0.20	0.48 $\pm$ 0.20	0.49 $\pm$ 0.04
b	0.27 $\pm$ 0.10	0.22 $\pm$ 0.06	0.04 $\pm$ 0.01
c	0.03 $\pm$ 0.004	0.07 $\pm$ 0.005	0.03 $\pm$ 0.004

An experiment was done in which rats were fasted for 24 hr. after periods of 1, 2 and 3 weeks on two regular feeding regimes and the liver glycogen estimated. The results are presented in Table 2 and indicate that it took at least 3 weeks for the animals

to settle down to these new conditions as judged by the fasting level of their liver glycogen.

Rats were kept for several months on the 1 hr. feeding regime and remained perfectly healthy. At autopsy they were found to have greatly enlarged stomachs but no other abnormality.

Liver glycogen levels after DNOC

The original histological observations of the livers packed with glycogen were made on tissues from rats which had been on continuous feeding and were killed after a series of daily doses of DNOC. Table 3 gives amounts of glycogen found in the

Table 3. *Effect of successive daily subcutaneous doses of DNOC on liver glycogen of rats*

(Daily dose of DNOC, 20 mg./kg. body wt. Rats were not fasted and were killed 24 hr. after the last injection of DNOC.)

No. of rats	No. of daily doses of DNOC	Liver glycogen (%)	
		With DNOC	Without DNOC
8	20	6.08 $\pm$ 0.21	3.24 $\pm$ 0.31
9	40	6.48 $\pm$ 0.36	2.86 $\pm$ 0.16

livers of rats on continuous feeding that had received a series of daily doses of DNOC compared with controls injected with saline. The rats were killed 24 hr. after the last injection of DNOC. The data confirm the histological observation that the non-fasting rat that has had previous injections of DNOC has more glycogen than normal in the liver. The immediate effect of a dose of DNOC is to reduce the amount of liver glycogen in the liver of the normal non-fasting rat (Table 4). It was, therefore, of interest to follow in more detail the changes that take place in the level of liver glycogen after injections of DNOC.

Table 4. *Effect of a single subcutaneous injection of DNOC on liver glycogen of rats*

(Dose of DNOC, 20 mg./kg. body weight. Rats were not fasted and were on the continuous feeding regime.)

Exp.	No. of rats	Time after DNOC (hr.)	Liver glycogen (%)	
			With DNOC	Without DNOC
1	3	0.5	0.78 $\pm$ 0.28	3.45 $\pm$ 0.16
2	4	1	0.73 $\pm$ 0.12	1.94 $\pm$ 0.29

It has been shown that the fasting liver glycogen is higher in rats accustomed to single feed regime than in those on continuous feeding. The effect of a series of injections of DNOC was to raise the fasting level in rats on either feeding regime. The animals were not given DNOC during the 24 hr. fasting period. The results from a number of experiments are collected and presented in Table 5. The

fasting liver glycogen was raised by a single injection and the effect was more marked after a series of 5-10 daily injections, but there was no significant increase in the fasting liver glycogen if the daily administration was carried on for longer periods which in some experiments extended to 40 days.

Table 5. *Effect of successive subcutaneous daily doses of DNOC on the liver glycogen of fasting rats*

(Rats received 10 daily doses of DNOC, 20 mg./kg. body weight. Rats fasted for 24 hr. after last dose of DNOC. The rats had been (a) on the continuous feeding regime, and (b) on the 1 hr. feeding regime.)

Feeding regime	No. of rats	Liver glycogen (%)
a With DNOC	17	0.59 $\pm$ 0.06
Without DNOC	47	0.085 $\pm$ 0.015
b With DNOC	33	3.34 $\pm$ 0.31
Without DNOC	53	0.70 $\pm$ 0.07

Observations were then made during the whole 24 hr. period on rats accustomed to a single meal regime. After 3 weeks on this regime some of the rats were given DNOC (20 mg./kg.) immediately after the feeding period for 10 successive days. Groups of rats were killed at different times after the final dose of DNOC. The results are given in Table 6. They show that in the animals that had

Table 6. *Effect of successive daily subcutaneous doses of DNOC in liver glycogen of rats during 24 hr. period following the last dose of DNOC*

(All rats on 1 hr. feeding regime. DNOC, 20 mg./kg. body weight given immediately after hourly feed. All treated rats given ten doses of DNOC.)

Time after DNOC (hr.)		No. of rats	Liver glycogen (%)
2	With DNOC	10	0.48 $\pm$ 0.16
	Without DNOC	15	3.96 $\pm$ 0.23
4	With DNOC	9	3.48 $\pm$ 0.83
	Without DNOC	10	4.97 $\pm$ 0.45
8	With DNOC	19	4.62 $\pm$ 0.59
	Without DNOC	18	7.16 $\pm$ 0.54
12	With DNOC	6	4.11 $\pm$ 0.72
	Without DNOC	8	8.82 $\pm$ 0.38
24	With DNOC	20	4.64 $\pm$ 0.43
	Without DNOC	20	0.84 $\pm$ 0.25

received DNOC the liver glycogen was much lower than in the control rats 2 hr. after the meal, but later it rose. It did not, however, exceed the level in the normal rats until sometime after 12 hr. but by the end of 24 hr. the differences were well marked. Since the rats receiving DNOC must have started their last meal with a liver glycogen level of between 2 and 4 %, it is probable that the low level 2 hr. after the meal (0.48 %) must have resulted from an in-

creased loss of glycogen from the liver and not from a failure to lay down more from products of digestion.

In other experiments the rats were killed at intervals between 24 and 48 hr. after the last dose of DNOC, having had a feed at the end of the first 24 hr. (Table 7). In these animals there was no fall in liver glycogen after the meal because no following DNOC had been given, but there was a greater quantity in the liver at 32 and 48 hr. in the DNOC-treated rats.

Table 7. *Effect of successive daily subcutaneous doses of DNOC on liver glycogen of rats during the period 24-48 hr. after the last doses of DNOC*

(Rats on 1 hr. feeding regime and given ten successive daily injections of DNOC (20 mg./kg.). Fed 24 hr. after the last dose of DNOC and killed at intervals thereafter.)

Time after DNOC (hr.)		No. of rats	Liver glycogen (%)
25	With DNOC	4	3.15 $\pm$ 0.14
	Without DNOC	4	2.02 $\pm$ 0.20
28	With DNOC	7	6.37 $\pm$ 0.46
	Without DNOC	8	4.55 $\pm$ 0.40
32	With DNOC	8	9.09 $\pm$ 0.13
	Without DNOC	8	5.97 $\pm$ 0.28
48	With DNOC	8	2.94 $\pm$ 0.37
	Without DNOC	8	0.41 $\pm$ 0.13

In one experiment the animals were killed 4, 8 and 24 hr. after their daily feed 7 days after receiving their last dose of DNOC. There was no difference in the glycogen levels in the control rats and those that had previously received DNOC.

Table 8. *Effect of a single injection of DNOC on the synthesis of liver glycogen of rats following glucose by mouth*

(Thirty-six rats fasted 24 hr. and then given 5 ml. 20% (w/v) glucose by mouth and divided into two groups of 18. One group were controls and the other injected subcutaneously with DNOC 20 mg./kg. body weight. These two groups were further subdivided and nine rats killed 2 and 4 hr. after receiving glucose.)

Time after glucose (hr.)	Liver glycogen (%)	
	With DNOC	Without DNOC
2	0.69 $\pm$ 0.21	1.54 $\pm$ 0.13
4	1.75 $\pm$ 0.17	1.49 $\pm$ 0.13

DNOC and glycogen synthesis in the liver of the fasting rat

The effect of DNOC on the synthesis of liver glycogen was examined in fasting rats that had been given glucose by mouth. DNOC was given immediately after the glucose and these rats, together with the controls given glucose and injections of saline, were killed 2 and 4 hr. later. The figures given in Table 8 show that there was no difference at

4 hr. in the amount of glycogen in the livers of the normal and poisoned rats. At 2 hr. there was still fluid in the stomachs of the DNOC rats while the stomachs of the normal rats were empty. Thus the lower level of glycogen in the liver at this time might partly be the result of poor absorption during the acute phase of poisoning.

DNOC and blood lactate

Cori & Cori (1928) found that after an injection of adrenaline the liver glycogen of rats fell rapidly but later rose above the starting level. They found a rise in the blood lactic acid in rats soon after the adrenaline had been given and suggested that lactic acid had been liberated from the muscles under the influence of the adrenaline, and that it was assimilated by the liver and converted into glycogen.

The blood sugar, blood lactic acid and liver glycogen were determined in rats killed at intervals after an injection of DNOC (20 mg./kg.). Although there was twice as much glycogen in the liver of those rats killed at 24 hr. that had received DNOC as in the controls, there had been no increase in the blood lactic acid levels of rats killed at 2, 4, 8, 12 and 16 hr. after the DNOC (Table 9).

Role of adrenals in the response to DNOC

The work of others (see below) has indicated that the rise in liver glycogen due to anoxia, alloxan diabetes and dietary changes may be mediated by

the adrenals. Attempts to detect the effects of DNOC on the deposition of liver glycogen in adrenalectomized rats were made difficult by the greater susceptibility of the adrenalectomized rats to the toxic action of DNOC. While most adrenalectomized rats could withstand one injection of 20 mg./kg., a second injection on the following day killed the majority of these animals. In two experiments on male rats the adrenalectomized rats were given 20 mg./kg. DNOC on the first day and 15 mg./kg. on the second day. There was a 20 % mortality but the survivors were killed 24 hr. later, not having been fasted. In both experiments the group that had received DNOC had a higher liver glycogen than the untreated adrenalectomized rats but in only one experiment was the difference significant.

The effect of a series of daily injections of DNOC on the weight of the adrenals was examined. Increases in adrenal weight have been reported in rats with alloxan diabetes where there is also an increase in the liver glycogen levels (Bennett & Koneff, 1946). Rats were given a series of twenty and forty daily injections of DNOC (20 mg./kg.) and at the end of the period they were killed and the adrenals removed, cleaned free of fat and weighed. Control rats were given daily injections of saline. The results are given in Table 10 and show that there was a significant increase in the weights of the adrenals of male rats receiving DNOC but not of the females.

Table 9. *The effect of a subcutaneous injection of DNOC (20 mg./kg. body weight) upon the levels of liver glycogen, blood sugar and blood lactic acid of rats*

(All animals on 1 hr. feeding regime and groups of four killed at intervals after feeding. Control rats had received no DNOC.)

Time after feed (hr.)	With DNOC			Without DNOC		
	Glycogen (%)	Blood sugar (mg./100 ml.)	Lactic acid (mg./100 ml.)	Glycogen (%)	Blood sugar (mg./100 ml.)	Lactic acid (mg./100 ml.)
2	0.63±0.21	166±19	22	4.95±0.46	106±2.8	29
4	2.23±0.29	91±10	22	5.88±0.62	48±3.5	22
8	3.77±0.47	118±3	21	9.49±1.25	99±5.5	22
12	5.29±0.67	123±3	24	8.85±0.45	94±2.5	23
16	4.31±0.85	109±3	21	6.38±1.10	96±7.0	20
24	5.56±0.48	76±1.5	25	2.74±0.60	70±5.5	21

Table 10. *The effect of repeated daily injections of DNOC on the adrenal weights of rats*

(Groups of male and female rats given 20 or 40 daily subcutaneous injections of 20 mg. DNOC/kg. and killed. Adrenal weights expressed as mg./100 g. body wt.)

Sex	Treated with DNOC			Controls		<i>t</i>	<i>P</i>
	No.	Wt. of adrenal (mg.)	No.	Wt. of adrenal (mg.)			
M.	22	22.46±0.73	24	19.72±0.59	3.02	<0.01*	
F.	9	35.28±1.64	8	32.56±1.24	1.24	<0.37†	

* Difference highly significant.

† Difference not significant.

t, ratio of an observed deviation to its estimated standard deviation.

P, the probability of obtaining a random sample more divergent than the group observed (Fisher, 1948).

DISCUSSION

An explanation of the effects of a change in the feeding habits on the fasting liver glycogen levels is suggested by the work of Tepperman, Brobeck & Long (1942-3). They studied the metabolic changes in rats submitted to hypophysectomy and they found marked changes in the respiratory quotient during the 24 hr. In considering the causes of these changes they realized that hypophysectomy rendered the rats hyperphagic so that they ate all the food as soon as it was presented to them. When normal rats were trained to eat all their food during 1 hr. a day, they found changes in the R.Q. similar to those in hyperphagic hypophysectomized rats. Werthessen (1937) had also found violent fluctuations in the R.Q. of rats fed only once daily.

Tepperman *et al.* (1942-3) concluded that in the normal animal there were three reserves of energy—the liver glycogen, the body fat and the food in the gut. Normally, a rat has food in its gut for the whole 24 hr., but in rats trained to eat all their food during 1 hr. a day the gut is empty for about 12 hr. These rats will be accustomed to a 12 hr. fast every day and so may be better adjusted to a 24 hr. fast than those used to feeding at will during the 24 hr.

These observations suggest that care may be necessary in assessing the effects of drugs, hormones or dietary changes upon the fasting liver glycogen levels. Before conclusions can be drawn about the significance of any differences that may be observed, it will be necessary to be certain that any endocrine imbalance, change in the palatability of the diet or effect of a drug has not modified the feeding habits of the treated animals.

The effect of DNOC was studied in rats accustomed either to continuous feeding or to a single daily feed and was similar in both groups. One injection of DNOC (20 mg./kg.) into a rat produces a profound toxic reaction lasting a few hours (Parker *et al.* 1951). These rats always have a higher liver glycogen level than control untreated rats when examined 24 hr. after an injection of DNOC. This difference is observed after single or repeated daily doses of DNOC in rats fasted or fed during the 24 hr. period after the DNOC.

On the basis of the observations of Tepperman *et al.* (1942-3) on rats trained to a single daily meal, it is possible that the higher liver glycogen might represent the response of the animal to the fact that its intestine is empty for 12 hr. each day, which might constitute a loss of an important reserve of energy and create a need to maintain a higher liver glycogen reserve.

Mackay & Drury (1941) have shown that the amount of energy reserves laid down as glycogen in 24 hr. is only one-tenth of the total consumed as food during the same period. In the fasting animal

it is the fat and in the fed animal the fat or intestinal contents that act as the main reserves of energy. Although the dose of DNOC may have some effect on the absorption of food from the gut, this effect was short-lived, for the fasted rat given glucose and then DNOC (20 mg./kg.) had laid down as much glycogen 4 hr. later as the untreated control (Table 8). It seems improbable, therefore, that the raised level of liver glycogen 24 hr. after a dose of DNOC can be attributed to an effect of the drug upon the absorption of food from the intestine with the imposition of an apparent fast upon the animal.

In recent years the influence of the adrenal upon the liver glycogen level has become well recognized. The effect that a number of entirely different procedures has upon the liver glycogen level has been attributed to an action on the adrenals. Mirski, Rosenbaum, Stein & Wertheimer (1938) found that the fasting level of glycogen was higher in rats receiving a diet of 70 % protein than in those receiving a diet of 70 % carbohydrate, a difference not seen in adrenalectomized rats. Evans (1934) found that a period of hypoxia increased the liver glycogen level in rats. This was not simply a reflexion of a slower rate of metabolism, because if the glycogen level was reduced by fasting at a normal atmospheric oxygen tension, it could then be raised by exposing the rats to a reduced oxygen tension. This effect is not seen in rats deprived of their adrenal cortex. Lewis, Thorn, Koepf & Dorrance (1942) showed that the effect of hypoxia on the liver glycogen was prevented by giving the animals 5 % carbon dioxide. Heilman (1944) found that the addition of carbon dioxide prevented the hypertrophy of the adrenal cortex that normally occurs after periods of hypoxia. He suggests that the addition of carbon dioxide prevents the acid-base shift that will result from the hyperpnoea which occurs at low oxygen tensions, and that a disturbance of the mineral metabolism causes a response in the adrenal cortex which incidentally results in changes in the liver glycogen level. These observations have been confirmed by Langley, Nims & Clarke (1950).

In alloxan diabetes Tuerkischer & Wertheimer (1946) and Weber (1946) reported that the rat has a liver glycogen level higher than normal. Weber (1946) found that this did not take place in adrenalectomized rats. Bennett & Koneff (1946) found that there was an increase in the weights of the adrenals in rats rendered diabetic with alloxan. Morita & Orten (1950) reported that there was no correlation between the height of the blood sugar and the amount of glycogen in the liver of diabetic rats.

In the experiments with DNOC it has proved impossible to establish with certainty whether the adrenals play a part in bringing about the changes in liver glycogen levels after DNOC. Although the

weight of the glands in male rats was increased by repeated daily injections of DNOC the increase was not significant in the case of the female rats. It proved impossible to give adrenalectomized rats repeated daily injections of DNOC in doses that in normal rats would have been adequate to produce the late rise in the liver glycogen.

A third possibility was that the rise in liver glycogen after the immediate toxic effects of DNOC had disappeared, might be explained on lines similar to those suggested by Cori & Cori (1928) to explain the late rise in liver glycogen after injecting adrenaline into rats. Loomis & Lipmann (1948) first showed that dinitrophenol inhibited the uptake of phosphate by respiring mitochondrial preparations. Judah & Williams-Ashman (1951) and Judah (1951) have extended these observations and shown that under the influence of dinitrophenol or DNOC the uptake of inorganic phosphate progressively decreases but the consumption of oxygen is increased. The increased respiration rate of animals poisoned with DNOC indicates a greater oxygen consumption and presumably increasing amounts of substrate are sacrificed to supply the energy needed for the increasingly inefficient phosphorylating processes. The sharp fall in the liver glycogen may be the reflexion of this demand for sources of energy.

When the acute phase of poisoning is over and the phosphorylating processes can again be carried out normally, there may well be an excess of metabolites remaining from the preceding period of increased oxidative activity. These may be taken up by the liver and converted into glycogen. Cori & Cori (1928) found a rise in the blood lactic acid after

adrenaline, but no rise in blood lactic acid occurred after DNOC. This does not invalidate the hypothesis, but a search has not been made for changes in the blood levels of other metabolites.

It is, of course, possible that any changes in the blood level might be too small to detect. This might be due to their slow release or to ability of the liver to remove them so efficiently that no accumulation could take place in the blood, but only an accumulation of the glycogen formed from them in the liver.

SUMMARY

1. The effects of single and repeated toxic doses of dinitro-*o*-cresol (DNOC) upon the liver glycogen of the rat are described.

2. An initial fall in liver glycogen is followed by a progressive rise, so that after 24 hr. the level is higher in control animals.

3. A single dose of DNOC did not impair the absorption of glucose or laying down of glycogen in fasting rats.

4. Blood lactic acid levels are not affected by the doses of DNOC given.

5. Repeated doses of DNOC increased the weight of the male adrenal but not that of the female rat.

6. The findings are discussed and the changes in liver glycogen are taken to be a reflexion of the general metabolic disturbance produced by DNOC.

7. The level of liver glycogen in a normal rat fasted for 24 hr. depends upon the feeding habits to which the animals were accustomed before the fast.

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