fragment and to CO_2 and water, 12 hr. after the injection of the hormone. In diabetic rats there is also a stimulation of the rate of two-carbon-fragment formation at this time interval, but no increase in the rate of oxidation of the two-carbon units.

4. It is suggested that the inhibition of fatty acid oxidation found 6 hr. after the injection is due to a release of insulin caused by the pancreatropic effect of the growth hormone, and not by the growth hormone itself. The effect of growth hormone is to produce an increased rate of fat degradation.

I wish to record my thanks to the Royal Society for an expenses grant.

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The Effect of Pituitary Growth Hormone and of Insulin on the Level of Oxidized and Reduced Coenzyme I in the Livers of Normal and Diabetic Rats

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The last few years have seen considerable advances in our knowledge of the mechanism of fat oxidation and synthesis. Reports from Green's laboratory (see Green, 1954), and from Lynen & Ochoa (1953), have contributed to the building-up of a composite picture in which acyl fatty acids are either shortened by 2-carbon atoms or lengthened by 2-carbon atoms as a result of four consecutive reactions, which together constitute the 'fatty acid cycle'. This cycle is envisaged as being completely reversible. Two of the reactions in the cycle involve hydrogen transfers, in one case by the flavin-adenine dinucleotide-linked enzyme, ethylene reductase, and in the other by the coenzyme I-linked enzyme, β -keto reductase. Lynen (1952-53) has suggested that the direction in which the cycle turns, i.e. whether it is a synthetic or a degradative cycle, depends on the availability of reduced coenzyme I (DPNH) as a source of hydrogen or of oxidized coenzyme I (DPN) to accept hydrogen. Indeed, he states: 'It would appear that in the living cell also the DPNH:DPN ratio may determine whether synthesis or degradation of the carbon chain will occur'. This suggestion has been verified by *in vitro* experiments (Hele & Popják, 1955), but not by *in vivo* experiments, where the experimental conditions are not so readily controlled. It seemed to us that the necessary *in vivo* conditions could best be obtained in animals

exposed to various hormonal influences which have previously been shown to favour either fat synthesis or breakdown. We have accordingly studied the DPN:DPNH ratio in insulin-treated rats, which are actively synthesizing fat, and in rats treated with pituitary growth hormone in which fat oxidation predominates. The results below indicate that although the DPN:DPNH ratios in diabetic and insulin-treated rats follow the predictions of Lynen, the ratio in growth-hormone-treated rats does not. In fact there appears to be little correlation between the cellular level of DPN or DPNH and the direction of the fatty acid cycle.

METHODS

Animals. The animals used were female hooded Norway rats aged 3-4 months, weighing 170-200 g. and maintained on stock diet (diet 41, Bruce & Parkes, 1946). In animals treated with growth hormone, the diet used was that described by Greenbaum (1953), which was supplied *ad lib.* before the commencement of treatment and at a daily level of 22 g. thereafter. Control rats for this group also received 22 g. of the special diet/day.

Diabetic animals were obtained by injecting 180 mg./kg. of body wt. of alloxan monohydrate dissolved as a 2% solution in a citrate-phosphate buffer, pH 4.0. The mortality rate was high, only about one-third of the rats surviving until the 21st day. The diabetic rats were kept for 3 weeks to ensure that there was no residual endogenous insulin. The surviving diabetic rats used in these experiments had

blood sugar levels (non-fasting) 400–600 mg./100 ml., and were excreting 7–12 g. of glucose/day.

Injection of hormones. The growth hormone used was a twice-crystallized preparation of the fraction *A* prepared by the method of Wilhelmi, Fishman & Russell (1948). The dose given was 1 mg. injected subcutaneously (into rats treated for 24 hr. or longer), intraperitoneally (for the groups being treated for 6 or 12 hr.), or intravenously when the treatment was for 1 hr. only. Control rats received similar injections of saline.

Insulin was a commercial sample of Wellcome insulin (Burroughs Wellcome and Co.). The dose of 0.25 unit was injected intravenously into the tail vein. Controls were similarly injected with saline.

Assay of DPN and DPNH. For assay of the coenzyme rats were killed by dislocation of the cervical vertebrae, and 500 mg. of the central lobe of the liver was rapidly removed and extracted as described by Eichel & Spirtes (1954). The time interval between killing and placing the liver sample into the heated medium was standardized at 1 min. The extract was then assayed as described by Eichel & Spirtes except that the volume of liquid used in extraction was reduced from 15 to 10 ml. in order to obtain more concentrated solutions for the spectrophotometric assay. We found, however, that when these extracts were centrifuged for 15 min. at 35 000 *g* as recommended by Eichel & Spirtes a clear supernatant was obtained, but a layer of floating particles made decantation impossible. A clear sample of the supernatant was obtained by withdrawing the middle layer with a capillary pipette. Measurements of the changes in extinction at 340 $\mu\mu$. were made on a Hilger Uvispek spectrophotometer.

Measurements were also made of the DPN and DPNH content of muscle in normal, growth-hormone-treated and insulin-treated rats. In these animals the quadriceps muscle was removed as rapidly as possible after death; 500 mg. was weighed on a torsion balance and then extracted and assayed in exactly the same way as liver.

RESULTS

The changes in the level of oxidized and reduced DPN in the liver of rats treated with pituitary growth hormone for periods of from 6 hr. to 7 days are shown in Table 1. Included in this table are the levels of coenzyme calculated per g. of liver, per total liver or total liver/100 g. of body weight. It will be seen that the DPN content per g. of liver remains unchanged throughout the 7 days of treatment, whereas the DPNH content increases as a result of the treatment. This increase may be noted as early as 6 hr., although it does not become statistically significant until the animal has been treated for 24 hr. The same tendency, an unchanged DPN and an increased DPNH, is also noticeable when the results are expressed as total oxidized or reduced coenzyme in the liver, although then the results are not statistically significant. More striking are the changes in the ratio DPN:DPNH. There was a fall in this ratio in all the treated groups, the fall being statistically significant as early as 6 hr. after the treatment and

remaining significantly lowered throughout the 7 days.

The very rapid change in the DPN:DPNH ratio made it seem worth while to investigate the effect of even shorter-term treatments of only 1 hr. For these, the injection was made into the tail vein. It was noted that the unavoidable handling of the rats during tail-vein injections invariably caused a lowering of both DPN and DPNH. To allow for this, all rats, control and experimental, were handled in as standard a manner as possible, and all were injected with the same volume of solution, saline in the controls, and growth hormone or insulin in the experimental rats. The results of this experiment are shown in Table 2. The handling seemed to affect the level of DPNH more than the level of DPN and this is reflected in a ratio of DPN:DPNH of 2.2 for control rats, a figure considerably higher than that found in normal controls. Again it will be noted that there is no change in the level of DPN, but there is a significant rise in the level of DPNH after treatment with growth hormone. In these animals the treatment with growth hormone brought about a synthesis of total coenzyme, since the combined DPN and DPNH of the treated animals is almost significantly greater than in the controls ($P = 0.055$). As an extension of the results shown in Table 1, treatment with growth hormone for only 1 hr. caused a significant fall in the DPN:DPNH ratio, the fall being even more striking than that obtained with long-term treatment.

In view of the pancreatropic effect of growth hormone, it was thought that these early effects might not necessarily be due to growth hormone itself, but could at least in part be due to the action of insulin, the secretion of which had been stimulated. A further experiment was therefore set up in which 0.25 unit of insulin was injected into the tail vein in the place of the growth hormone. The results of this experiment are also given in Table 2. In contrast to growth hormone, insulin caused an unequivocal increase in both DPN and DPNH, the level of reduced coenzyme being almost doubled. The total coenzyme is also significantly increased over control level, implying an actual synthesis of coenzyme even in so short a period as 1 hr. The ratio DPN:DPNH was exceedingly low, and was in fact the lowest encountered in the present series of experiments. This result did little towards answering the question whether the very quick response of the DPN:DPNH ratio after growth hormone was due to a direct action of the hormone or was due to an indirect response via insulin. To resolve this question use was made of a series of diabetic rats in which an insulin effect would not be possible. The results of this experiment are given in Table 3. The effect of diabetes is to cause a profound

Table 1. Level of oxidized and reduced coenzyme I in the livers of rats treated with anterior-pituitary growth hormone

Time of treatment	No. of animals	Coenzyme I/g. wet wt. of liver			Total liver coenzyme I			Total liver coenzyme I/100 g. body wt.		
		DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	DPN : DPNH	DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)
Control	6	399 $\pm$ 16.2	213 $\pm$ 12.6	612 $\pm$ 25.3	1.91 $\pm$ 0.09	3242 $\pm$ 211	1727 $\pm$ 139	4969 $\pm$ 330	1596 $\pm$ 94	853 $\pm$ 80
	6 hr.*	387 $\pm$ 10.3	238 $\pm$ 8.8	625 $\pm$ 17.6	1.63 $\pm$ 0.05	3034 $\pm$ 186	1863 $\pm$ 116	4897 $\pm$ 294	1529 $\pm$ 68	941 $\pm$ 46
12 hr.*	6	379 $\pm$ 14.6	239 $\pm$ 4.8	618 $\pm$ 4.36	1.59 $\pm$ 0.07	2929 $\pm$ 117	1853 $\pm$ 73	4782 $\pm$ 146	1456 $\pm$ 58	921 $\pm$ 30
	1 day†	401 $\pm$ 8.5	256 $\pm$ 4.6	657 $\pm$ 9.04	1.56 $\pm$ 0.04	3202 $\pm$ 121	2058 $\pm$ 112	5260 $\pm$ 223	1580 $\pm$ 63	1014 $\pm$ 50
3 days†	6	389 $\pm$ 19.2	282 $\pm$ 11.5	651 $\pm$ 27.8	1.49 $\pm$ 0.05	3035 $\pm$ 127	2043 $\pm$ 69	5078 $\pm$ 171	1466 $\pm$ 67	987 $\pm$ 38
	7 days†	412 $\pm$ 24.9	250 $\pm$ 9.9	662 $\pm$ 17.8	1.65 $\pm$ 0.04	3268 $\pm$ 56	1983 $\pm$ 51	5251 $\pm$ 40	1565 $\pm$ 40	950 $\pm$ 28
		P=0.392	P=0.089	P=0.846	P=0.024	P=0.224	P=0.444	P=0.771	P=0.923	P=0.629
		P=0.923	P=0.011	P=0.143	P=0.009	P=0.844	P=0.060	P=0.502	P=0.300	P=0.698
		P=0.698	P=0.034	P=0.343	P=0.004	P=0.444	P=0.077	P=0.771	P=0.762	P=0.199
		P=0.700	P=0.069	P=0.148	P=0.060	P=0.923	P=0.148	P=0.447	P=0.047	P=0.001

* Intraperitoneal injection.

† Subcutaneous injection.

P = probability that difference from control value is due solely to chance.

Table 2. Effect of very short-term treatment of normal rats with pituitary growth hormone and insulin on the level of oxidized and reduced coenzyme I

Time of treatment	No. of animals	Coenzyme I/g. wet wt. of liver			Total liver coenzyme I			Total liver coenzyme I/100 g. body wt.		
		DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	DPN : DPNH	DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)
Control	6	349 $\pm$ 7.8	162 $\pm$ 3.7	511 $\pm$ 18.7	2.20 $\pm$ 0.13	2514 $\pm$ 167	1165 $\pm$ 102	3679 $\pm$ 204	1338 $\pm$ 24	620 $\pm$ 39
	6	358 $\pm$ 11.9	214 $\pm$ 12.0	572 $\pm$ 21.0	1.69 $\pm$ 0.09	2808 $\pm$ 50	1678 $\pm$ 86	4484 $\pm$ 112	1542 $\pm$ 33	921 $\pm$ 49
Injected with growth hormone 1 hr.	6	406 $\pm$ 9.1	306 $\pm$ 22.5	712 $\pm$ 26.8	1.36 $\pm$ 0.09	2914 $\pm$ 166	2177 $\pm$ 155	5091 $\pm$ 270	1553 $\pm$ 88	1155 $\pm$ 65
	6	406 $\pm$ 9.1	306 $\pm$ 22.5	712 $\pm$ 26.8	1.36 $\pm$ 0.09	2914 $\pm$ 166	2177 $\pm$ 155	5091 $\pm$ 270	1553 $\pm$ 88	1155 $\pm$ 65
Injected with insulin 1 hr.	6	406 $\pm$ 9.1	306 $\pm$ 22.5	712 $\pm$ 26.8	1.36 $\pm$ 0.09	2914 $\pm$ 166	2177 $\pm$ 155	5091 $\pm$ 270	1553 $\pm$ 88	1155 $\pm$ 65
	6	406 $\pm$ 9.1	306 $\pm$ 22.5	712 $\pm$ 26.8	1.36 $\pm$ 0.09	2914 $\pm$ 166	2177 $\pm$ 155	5091 $\pm$ 270	1553 $\pm$ 88	1155 $\pm$ 65
		P=0.001	P=0.001	P=0.001	P=0.001	P=0.105	P=0.001	P=0.001	P=0.047	P=0.001
		P=0.001	P=0.001	P=0.001	P=0.001	P=0.105	P=0.001	P=0.001	P=0.047	P=0.001

Table 3. Levels of oxidized and reduced coenzyme I in the livers of diabetic rats and diabetic rats treated with pituitary growth hormone

Treatment	No. of animals	Coenzyme I/g. wet wt. of liver			Total liver coenzyme I			Total liver coenzyme I/100 g. body wt.		
		DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	DPN : DPNH	DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)	Total ($\mu\text{g.}$)	DPN ($\mu\text{g.}$)	DPNH ($\mu\text{g.}$)
Diabetic controls	6	346 $\pm$ 17.4	99.8 $\pm$ 5.8	446 $\pm$ 78	3.48 $\pm$ 0.06	2808 $\pm$ 80	799 $\pm$ 27	3607 $\pm$ 103	1802 $\pm$ 106	511 $\pm$ 34
	6	427 $\pm$ 19.9	190 $\pm$ 7.2	617 $\pm$ 26.8	2.24 $\pm$ 0.04	3373 $\pm$ 212	1502 $\pm$ 75	4875 $\pm$ 287	2334 $\pm$ 142	1040 $\pm$ 51
Diabetic plus growth hormone 1 hr.	6	427 $\pm$ 19.9	190 $\pm$ 7.2	617 $\pm$ 26.8	2.24 $\pm$ 0.04	3373 $\pm$ 212	1502 $\pm$ 75	4875 $\pm$ 287	2334 $\pm$ 142	1040 $\pm$ 51
	6	427 $\pm$ 19.9	190 $\pm$ 7.2	617 $\pm$ 26.8	2.24 $\pm$ 0.04	3373 $\pm$ 212	1502 $\pm$ 75	4875 $\pm$ 287	2334 $\pm$ 142	1040 $\pm$ 51
		P=0.015	P=0.001	P=0.065	P=0.001	P=0.034	P=0.001	P=0.003	P=0.015	P=0.001
		P=0.015	P=0.001	P=0.065	P=0.001	P=0.034	P=0.001	P=0.003	P=0.015	P=0.001

Table 4. *Levels of oxidized and reduced coenzyme I in the quadriceps muscle of normal rats treated for 1 hr. with growth hormone (0.5 mg.) or insulin (0.25 unit)*

Treatment	No. of animals	Coenzyme I/g. wet wt. of muscle			
		DPN (μ g.)	DPNH (μ g.)	Total (μ g.)	DPN:DPNH
Control	8	284 $\pm$ 13.5	46 $\pm$ 3.14	330 $\pm$ 11.0	6.48 $\pm$ 0.69
Growth hormone	6	292 $\pm$ 5.7	36 $\pm$ 2.63	328 $\pm$ 8.0	8.35 $\pm$ 0.40
Insulin	6	273 $\pm$ 12.6	37 $\pm$ 2.11	310 $\pm$ 13.5	7.45 $\pm$ 0.45

lowering of the level of DPNH, and a very much smaller change in the DPN as compared with levels in the handled controls (shown in Table 2). As a consequence the DPN:DPNH ratio rises to 3.48. Treatment of these diabetic animals with growth hormone for 1 hr. causes a rise of both DPN and DPNH to normal levels with a more normal ratio of 2:2.

In addition to the experiments described above, a few measurements were made of the levels of DPN and DPNH in the quadriceps muscle. Table 4 presents a summary of the results of these experiments. It is noticeable that while the level of DPN is about 80% of that found in the liver the DPNH is only about 30%; the ratio DPN:DPNH in muscle is 6.48 as compared with a normal ratio of 2:2 in liver. It was found, however, that with muscle, unlike liver, the level of DPNH was extremely variable, and this, together with the inaccuracies inherent in the measurement of such small values, makes these results of doubtful significance. Even lower values of muscle DPNH have been reported by Jedeikin & Weinhouse (1955). These factors make it difficult to assess whether either growth hormone or insulin has any effect on muscle coenzyme I levels.

DISCUSSION

Treatment of normal rats with insulin caused an increase in both DPN and DPNH, the latter being almost doubled. The DPN:DPNH ratio was decreased to 1.36. Diabetic rats, on the other hand, showed no change in the level of DPN, but a profound fall in DPNH to almost half of that found in the control rats. The ratio of DPN:DPNH in diabetic animals was 3.48. These results are entirely in agreement with those reported by Helmreich, Holzer, Lamprecht & Goldschmidt (1954). The changes in reduced coenzyme I could be accounted for largely in terms of the effect of the hormone on glycolysis, which is probably a major source of DPNH, in that the injection of insulin increases glycolysis whereas deprivation of the hormone decreases it. With insulin we have a situation which appears to conform with the view of Lynen (1952-53) that a high level of DPNH is necessary for fatty acid carbon-chain elongation. Osborn,

Chaikoff & Felts (1951) have shown that in the absence of insulin fat synthesis is low, while the presence of the hormone increases the rate of carbon incorporation into fatty acids fivefold. In the present series of experiments we have found that diabetic rats have a preponderance of DPN, as evidenced by a DPN:DPNH ratio of 3.48, and a decreased availability of hydrogen for fat synthesis. On the other hand, rats treated with insulin have a DPN:DPNH ratio of 1.36 and hence have an increased capacity for fat synthesis. This is also in agreement with the views of Helmreich *et al.* (1954).

In rats treated with pituitary growth hormone, the correlation between the ratio DPN:DPNH and fat metabolism is not so clear. The injection of growth hormone for periods as short as 1 hr. or as long as 7 days uniformly results in an increase of DPNH and in a decreased DPN:DPNH ratio, conditions which on the Lynen formulation should favour fat synthesis. It is well established, however, that rats treated with growth hormone over the same time intervals have a greatly increased rate of fat oxidation (Greenbaum & McLean, 1953) and a reduced rate of fat synthesis (Greenbaum & Glascock, unpublished work). If the hydrogen of DPNH represents the reductive potential necessary for fat synthesis then clearly the hydrogen must be generated outside the fatty acid cycle. According to Recant (1952) glycolysis does not appear to be affected by growth hormone, and an increased glycolysis can therefore be discarded as a source of hydrogen. It seems much more likely that the increased DPNH in growth-hormone-treated rats represents not so much a potential for fat synthesis but simply a result of fat degradation. Each turn of the fatty acid cycle will lead to the reduction of 1 molecule of DPN by β -keto reductase. Thus we might expect a rise in the DPNH in conditions of increased fat degradation, conditions known to exist in growth-hormone-treated rats. If this is true the level of DPNH in the liver of growth-hormone-treated rats does not influence the direction of the fatty acid cycle, but is simply a reflexion of the way it is turning. In this context the increase in DPNH found in growth-hormone-treated diabetic animals is of interest. The diabetic rat has a much reduced DPNH owing to the failure

of glycolysis, and the injection of growth hormone restores the level to normal. This effect is not mediated by insulin, and there is no reason to believe it is due to any effect on glycolysis itself. It therefore seems most probable that here the source of hydrogen for the reduction of DPN is from an increased fat catabolism.

Helmreich *et al.* (1954) have studied the relation of liver DPNH and ketosis in starvation and in diabetes, and have concluded that DPNH has an antiketogenic affect in these animals. This relationship does not appear to apply to the ketosis induced by pituitary growth hormone. Liver slices from rats treated for 24 hr. with growth hormone produce more than twice as much acetoacetate, with oleate as substrate, than do untreated controls (Greenbaum & McLean, 1953), yet at this time interval the level of DPNH is significantly raised over the control level (Table 1), which is inexplicable in terms of the scheme of Helmreich *et al.* (1954), but readily explained in terms of the views advanced above.

The foregoing results are difficult to reconcile with Lynen's (1952-53) suggestion that the rate of fat synthesis is determined by the level of DPNH. Although it seems most probable that a low DPN:DPNH ratio is necessary for fat synthesis, the extension of this, that a low DPN:DPNH ratio would determine the direction of the fatty acid cycle in favour of carbon-chain elongation, does not seem fully justified. There appear to be factors other than the availability of hydrogen which also exert some influence on the direction in which the cycle works. The mechanism by which growth hormone influences fat metabolism in favour of degradation is being further investigated.

SUMMARY

1. Changes in the levels of oxidized coenzyme I (DPN) and reduced coenzyme I (DPNH) have been studied in the liver of (a) normal, (b) growth-

hormone-treated normal, (c) insulin-treated normal, (d) diabetic and (e) growth-hormone-treated diabetic rats, with periods of hormone treatment ranging from 1 hr. to 7 days.

2. Growth hormone has little effect on the DPN of normal rats but increases the level of DPNH. This effect is manifest at even the shortest time intervals.

3. Insulin increases both the DPN and DPNH, the latter to a greater extent.

4. Diabetes causes a profound fall in DPNH but this can be completely restored in 1 hr. by the injection of growth hormone.

5. The relation of these changes to the rate of fat synthesis in hormonally treated rats is discussed.

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