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Hormones and Liver Cytoplasm

2. ADENOSINE TRIPHOSPHATASE, GLUCOSE 6-PHOSPHATASE AND XANTHINE OXIDASE AS AFFECTED BY HYPOPHYSECTOMY, GROWTH-HORMONE TREATMENT AND ADRENALECTOMY*

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The yield of the mitochondrial fraction obtained from rat liver by differential centrifuging is decreased after adrenalectomy, and also after hypophysectomy unless growth hormone is administered; on the other hand, the yield of the microsomal fraction is increased after hypophysectomy, whether or not growth hormone is given (Reid, 1955, 1956). It cannot be assumed that there are parallel changes in the amounts of the enzymes characteristically present in the different cytoplasmic elements. Data bearing on this question are presented here and in the following paper (Stevens & Reid, 1956).

The enzymes adenosine triphosphatase (ATPase), glucose 6-phosphatase and xanthine oxidase have been selected for study, because of their different locations in normal cytoplasm and also because of their possible intrinsic interest in relation to hormonal control of liver metabolism.

EXPERIMENTAL

Treatment of animals

The experiments were performed with young albino male rats, which were fed on a protein-rich diet in restricted amount and were studied 1½–3 weeks after any operative

procedure. Treatment with the growth-hormone preparation, which was virtually free of thyrotrophin, was given for 8 days at a daily dose of 0.3 mg., as described in a previous paper (Reid, O'Neal, Stevens & Burnop, 1956) in which fuller details of the general procedures are given. Some experiments were performed with rats given thiouracil or thyroxine as in the experiments of Glock & McLean (1955).

To minimize the effect of any day-to-day variations in environment or technique, the values for experimental rats were not directly averaged, but were compared with those for control rats (intact or sham-operated) studied simultaneously, the differences being averaged. At least six untreated hypophysectomized rats, five hypophysectomized rats given growth hormone and six adrenalectomized rats were used in the study of each enzyme.

Isolation of tissue fractions

The procedures for isolation were, unless otherwise stated, as described previously (Reid *et al.* 1956). One modification, which was introduced with the aim of stabilizing glucose 6-phosphatase (Beaufay, Hers, Berthet & Duve, 1954), was the addition of ethylenediaminetetraacetic acid (EDTA; 0.001 M) to the isotonic sucrose solution other than that used for the preliminary perfusion of the liver.

The 'nuclear fraction' was discarded, and the supernatant 'cytoplasmic fraction' was further centrifuged to give a mitochondrial fraction (which was washed once, without removal of the 'fluffy layer'), a microsomal fraction and a supernatant fraction. These centrifugings were performed in an angle-head centrifuge under conditions which have been described elsewhere (Reid, 1955, 1956), the final centrifuging being at 20 000 g for 90 min. The mitochondrial and microsomal fractions were resuspended in the sucrose medium. Portions of each isolated fraction were taken for certain of the enzymic assays, as indicated below; the remainder was frozen by immersion in a mixture of acetone and solid CO₂, and was stored at -30°.

* The paper by Reid, O'Neal, Stevens & Burnop (1956) is to be regarded as Part 1.

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Enzymic assays

The conditions used in the enzymic assays were such that the activity was linearly related to the amount of tissue and the time of incubation. Each assay for ATP-ase or glucose 6-phosphatase was done in duplicate.

ATP-ase. The assays were performed essentially as described by Lardy & Wellman (1953), at pH 7.4 in the presence of Mg^{2+} ions (0.002M). Incubation was carried out for 15 min. at 30° with shaking, after addition of neutralized ATP (to 0.003M; disodium salt, '99% ATP', as supplied by Nutritional Biochemicals Corporation). The supernatant fluid obtained after addition of trichloroacetic acid solution and centrifuging was analysed for orthophosphate as described by King (1951), with ascorbic acid as the reducing agent. Novikoff, Hecht, Podber & Ryan (1952) and Kielley & Kielley (1953) have shown that the orthophosphate liberated during the incubation is a valid measure of ATP-ase activity. In agreement with Novikoff *et al.* the activity of the whole cytoplasmic fraction was quantitatively recovered in the subfractions derived therefrom.

The assays for 'free' ATP-ase were made in the presence of sucrose (0.25M), with freshly isolated mitochondrial fractions. 'Free' ATP-ase activity is known to be unaffected by the addition of Mg^{2+} ions (Witter, Watson & Cottone, 1955). Measured samples of these and other fractions were frozen and thawed after dilution with water, to liberate the 'total' ATP-ase activity (Novikoff *et al.* 1952; Witter *et al.* 1955).

Glucose 6-phosphatase. The assays were carried out essentially according to Duve, Berthet, Hers & Dupret (1949), in the presence of cacodylate buffer (pH 6.5) and of EDTA (0.001M; Beaufay *et al.* 1954). The procedures for incubation and for estimation of liberated orthophosphate were as described above; the substrate (0.0042M) was the dibarium salt of glucose 6-phosphate (Nutritional Biochemicals Corporation).

Hers, Berthet, Berthet & Duve (1951) have emphasized that the true recovery of glucose 6-phosphatase activity in isolated fractions is difficult to determine, because of the presence of an inhibitor in the supernatant fraction and because of the lability of the enzyme. In the present experiments, the apparent recovery of activity with different sets of fractions (each set including fractions derived from a

control rat) ranged from 58 to 92% but varied little within each set. The hormonal effects reported below could be demonstrated either with freshly prepared fractions or with fractions which had been frozen and stored (as used for some of the assays). With microsomal fractions, freezing may entail some loss of activity (Beaufay & Duve, 1954a). It has now been found that this loss does not exceed 20% with whole cytoplasmic fractions, but may be substantial with microsomal fractions, treatment of which with deoxycholate (0.05%; Beaufay & Duve, 1954b) does not restore the activity lost after freezing. Accordingly, the values reported below for the glucose phosphatase activity of microsomal fractions should be regarded as minimal figures.

Xanthine oxidase. The assay depended on the spectrophotometric determination, at 295 $m\mu$, of uric acid formed from xanthine, essentially under the conditions described by Kalckar (1947a, b). A measured sample of the freshly isolated fraction was incubated for 1 hr. at 37° in the presence of an equal volume of pyrophosphate buffer (0.1M; pH 8.0) and was finally centrifuged for 30 min. at 20 000 g. The supernatant fluid, after storage overnight in the cold, was assayed in the presence of xanthine (0.00025M) in a silica cell at 24°, in a Unicam SP. 500 spectrophotometer with water circulating at a constant temperature through the cell carriage. The optical densities of the contents of this cell and of a control cell (without xanthine) were compared with that of a blank cell (without tissue), for 10 min. at intervals of 1 min. The average net changes in optical density were determined graphically. The 'endogenous activity', which was measured by comparison of the control cell with the blank cell and was subtracted from the total activity, was very low and showed no dependence upon the hormonal status of the animal.

RESULTS

ATP-ase. As shown in Table 1, hypophysectomy led to a significant decrease, which could be prevented by growth hormone, in the ATP-ase activity of the microsomal fraction, but not in that of the cytoplasm as a whole. Adrenalectomy led to a decrease in the ATP-ase activity, both of the

Table 1. *ATP-ase (Mg^{2+} -activated) activity in relation to hormonal status*

Activities ('total', unless otherwise stated) are expressed as μ g. of P liberated/min. by an amount of tissue corresponding to 100 g. body wt. In this and subsequent tables, the standard error is given after each mean difference, and the number of degrees of freedom and the probability that the difference could be due to chance are given parenthetically.

	Control rats: mean value	Experimental rats: difference from corresponding control rats		
		Hypophysectomized	Hypophysectomized + growth hormone	Adrenalectomized
Whole cytoplasmic fraction	1.30	-0.21 $\pm$ 0.184 (8)	-0.27 $\pm$ 0.175 (6)	-0.39 $\pm$ 0.144 (12; $P < 2.5\%$)
Mitochondrial fraction	0.93	-0.11 $\pm$ 0.113 (8)	-0.15 $\pm$ 0.153 (6)	-0.17 $\pm$ 0.109 (12)
Microsomal fraction	0.34	-0.075 $\pm$ 0.020 (7; $P < 1\%$)	+0.005 $\pm$ 0.018 (6)*	-0.08 $\pm$ 0.030 (12; $P < 5\%$)
Supernatant fraction	0.11	-0.013 $\pm$ 0.025 (7)	-0.037 $\pm$ 0.040 (6)	0 $\pm$ 0.006 (8)
Mitochondrial fraction, 'free' activity	0.17	-0.015 $\pm$ 0.016 (6)	-0.005 $\pm$ 0.016 (3)	-0.06 $\pm$ 0.031 (7)

* Difference from corresponding untreated hypophysectomized rats = +0.075 $\pm$ 0.027 (5; $P < 5\%$).

whole cytoplasm and of the microsomal fraction. Neither with hypophysectomy nor with adrenalectomy did the mitochondrial and supernatant fractions show consistent decreases in activity.

In some experiments, determinations were made not only of the 'total' ATP-ase activity, but also of the 'free' ATP-ase activity of freshly isolated mitochondrial fractions assayed in the presence of sucrose (0.25 M). This activity showed no dependence on the hormonal status of the animals (Table 1).

Glucose 6-phosphatase. The assays for glucose 6-phosphatase (Table 2) showed a significant decrease after hypophysectomy in the case of the whole cytoplasm, the microsomal fraction and the supernatant fraction. The decrease in the cytoplasmic level after hypophysectomy could be partially reversed by the administration of growth hormone. No significant changes in the level of the enzyme were found after adrenalectomy.

Xanthine oxidase. The activity of this enzyme in the whole cytoplasmic fraction was markedly raised by hypophysectomy, whether or not growth hormone had been given, and was decreased by adrenalectomy (Table 3). With the supernatant fraction, in which 70 % of the cytoplasmic activity was recovered, similar changes were observed. In no instance was xanthine oxidase activity detected in the mitochondrial or microsomal fractions.

Since the effect of hypophysectomy on xanthine oxidase activity could not be attributed to deprivation of growth hormone or of adrenocorticotrophin, the possible role of thyrotrophin was investigated by ascertaining whether thiouracil or thyroxine could alter the level of activity in intact rats. As is shown in Table 3, no significant changes in xanthine oxidase activity followed the induction of hypothyroidism or hyperthyroidism by these agents.

DISCUSSION

In attempting to assess the metabolic implications of the changes in enzymic activity now observed with the whole cytoplasm, it should be kept in mind that the changes were usually relatively small (about 30 %) and that the rates of the reactions catalysed by the enzymes studied might be limited *in vivo* by the amount of substrate rather than of enzyme.

The function of ATP-ase in liver cytoplasm is debatable (Ernster, Ljunggren & Lindberg, 1954). It is possible that microsomal ATP-ase, the data for which are considered below, plays a role in the release of energy for cytoplasmic fibrillar activity (Abood & Romanchek, 1955).

It is well established that hypophysectomized animals readily develop hypoglycaemia during

Table 2. *Glucose 6-phosphatase activity in relation to hormonal status*

Activities are expressed as $\mu\text{g. of P liberated/min.}$ by an amount of tissue corresponding to 100 g. body wt. (See also Table 1.)

	Control rats: mean value	Experimental rats: difference from corresponding control rats		
		Hypophysectomized	Hypophysectomized + growth hormone	Adrenalectomized
Whole cytoplasmic fraction	0.66	-0.20 $\pm$ 0.048 (8; $P < 0.5\%$)	-0.09 $\pm$ 0.071 (6)*	+0.07 $\pm$ 0.052 (7)
Mitochondrial fraction	0.13	-0.06 $\pm$ 0.035 (4)	-0.02 $\pm$ 0.042 (3)	-0.06 (1)
Microsomal fraction	0.21	-0.045 $\pm$ 0.013 (8; $P < 2.5\%$)	-0.035 $\pm$ 0.029 (5)	-0.02 $\pm$ 0.022 (7)
Supernatant fraction	0.12	-0.06 $\pm$ 0.010 (4; $P < 0.5\%$)	-0.03 $\pm$ 0.004 (3; $P < 1\%$)	-0.01 (1)

* Difference from corresponding untreated hypophysectomized rats = +0.085 $\pm$ 0.342 (7; $P < 5\%$).

Table 3. *Xanthine oxidase activity in relation to hormonal status*

Activities are expressed as the increase in optical density which would be produced in 1 min. by an amount of tissue corresponding to 100 g. body wt. A value of unity corresponds to the oxidation of 0.104 μmoles of xanthine to uric acid. (See also Table 1.)

(See also Table 1.)		Experimental rats: difference from corresponding control rats				
	Control rats: mean value	Hypophysectomized	Hypophysectomized + growth hormone	Adrenalectomized	Hypothyroid	Hyperthyroid
Whole cytoplasmic fraction	3.08	+1.09 ± 0.116 (7; <i>P</i> < 0.1%)	+0.85 ± 0.260 (6; <i>P</i> < 0.5%)	-0.76 ± 0.290 (13; <i>P</i> < 2.5%)	+0.07 ± 0.654 (5)	-0.79 ± 0.485 (5)
Supernatant fraction	2.14	+0.81 ± 0.167 (7; <i>P</i> < 0.5%)	+0.58 ± 0.268 (7)	-0.76 ± 0.282 (13; <i>P</i> < 2.5%)	+0.68 ± 0.337 (5)	-0.58 ± 0.380 (5)

fasting unless growth hormone is given before the fast (Engel, 1954). This phenomenon could be partly explained by the present observation that hypophysectomy leads to a fall in glucose 6-phosphatase activity which can be partially reversed by growth hormone.

The effectiveness of growth hormone in restoring glucose 6-phosphatase activity need not be regarded as incompatible with its ineffectiveness in intact rats in the experiments of Langdon & Weakley (1955). However, it is difficult to explain why Ashmore, Hastings, Nesbitt & Renold (1956) found hypophysectomy to be without effect on glucose 6-phosphatase activity, calculated as the concentration in fresh liver tissue. Recalculation of the present data on this basis shows a decrease of 46% ($P < 0.5\%$) after hypophysectomy, in agreement with a recent report by Harper (1956), who was, however, unable to demonstrate any reversal of the effect by growth hormone.

If xanthine oxidase activity is indeed a measure of uric acid synthesis, it would appear that this process is slowed by adrenalectomy and stimulated by hypophysectomy. Experiments reviewed by Christman (1952) suggest that the process can likewise be stimulated by the administration of adrenocorticotrophin or cortisone. It is conceivable that with a change in the rate of catabolism of xanthine there is a concomitant change in that of ribonucleic acid, but there is, in fact, no definite evidence that the catabolism of the latter changes after hypophysectomy or adrenalectomy (Stevens & Reid, 1956).

The finding that xanthine oxidase activity is not significantly influenced by growth hormone is in agreement with experiments performed with intact rats by Remy, Richert, Westerfeld & Tepperman (1950). In confirmation of other experiments by these authors, the activity has shown little dependence on thyroid status. There is, then, no evidence that the effect of hypophysectomy is attributable to deprivation of growth hormone, adrenocorticotrophin or thyrotrophin.

The data for the distribution of enzymic activity among the separated fractions, as distinct from the level in the whole cytoplasm, may be considered in relation to the suggestion of Weiss (1955) that an increase in the fragility of cytoplasmic particles could account for certain hormonal effects (Reid, 1955, 1956), such as the diminution in the yield of the mitochondrial fraction after hypophysectomy or adrenalectomy and in the amount of ribonucleic acid in that fraction after hypophysectomy. Such an increase in the fragility of mitochondria might be reflected in an apparent shift of enzymes from this fraction into the microsomal or supernatant fraction, as occurs after adrenalectomy with D-amino acid oxidase (Kuriaki & Kensler, 1954). Another

possible consequence of an increase in mitochondrial fragility is an increase in 'free' ATP-ase (supposedly an index of mitochondrial integrity) at the expense of 'latent' ATP-ase.

No such increase in 'free' ATP-ase has now been found after hypophysectomy or adrenalectomy; but it has become apparent that this activity is not in fact a satisfactory guide to the morphological integrity of mitochondria (Witter *et al.* 1955; Birbeck & Reid, 1956). Moreover, there was no indication of a shift of ATP-ase activity from the mitochondrial fraction to the microsomal or supernatant fraction; indeed, the activity in the microsomal fraction fell after hypophysectomy (unless growth hormone had been administered) or after adrenalectomy.

These changes in microsomal ATP-ase activity must be considered in relation to the current view that ATP-ase activated by Mg^{2+} ions is a mitochondrial enzyme. The activity of microsomal fractions is low in relation to that of mitochondrial fractions (Schneider, Hogeboom & Ross, 1950; Novikoff *et al.* 1952) and could perhaps be attributed to the presence of mitochondrial fragments (Kielley & Kielley, 1953), although not of intact mitochondria, which were absent from microsomal fractions examined by electron microscopy in the present study (Birbeck & Reid, 1956). A preliminary experiment has indicated that the pH optimum of microsomal ATP-ase is, in fact, similar to that of mitochondrial ATP-ase (pH 8.5), although perhaps less sharp. However, no reduction in the microsomal ATP-ase activity of normal rats can be achieved with the use of a new homogenizing medium, which is superior to sucrose solution in preserving the morphological integrity of mitochondria (Birbeck & Reid, 1956). Moreover, recent studies with particulate fractions from liver cytoplasm have suggested that there exist two distinct ATP-ases activated by Mg^{2+} ions (Thomson & Mikuta, 1954; Demers, D'Iorio & Robillard, 1955; Abood & Romanchek, 1955). It is then unlikely that mitochondrial fragments are the site of the ATP-ase activity which has now been found in microsomal fractions and which is peculiarly susceptible to hormonal influences.

Not only the ATP-ase activity, but also the glucose 6-phosphatase activity of the microsomal fraction is diminished after hypophysectomy, although hypophysectomy is known to increase the yield of this fraction (Reid, 1955, 1956). The proportion of the cytoplasmic glucose 6-phosphatase activity recovered in the microsomal fraction was low in comparison with that reported by Hers *et al.* (1951), the conditions in the present experiments being such that the mitochondrial fractions contained a relatively high proportion of the activity and that some of the microsomal fractions may

have undergone partial inactivation. However, the distribution of the enzyme appeared not to be dependent on hormones.

The distribution of xanthine oxidase likewise appeared to be uninfluenced by alterations in hormonal balance. Most of the cytoplasmic activity was recovered in the supernatant fraction and none was found in the particulate fractions, in accordance with previous findings (Meikleham, Wells, Richert & Westerfeld, 1951; Villela, Mitidieri & Affonso, 1955; Morell, 1955).

Further consideration is given in the next paper (Stevens & Reid, 1956) to the interpretation of the data for enzyme distribution among the fractions separated from liver cytoplasm.

SUMMARY

1. The levels of activity of adenosine triphosphatase (ATP-ase) activated by Mg^{2+} ions, glucose 6-phosphatase and xanthine oxidase in rat-liver cytoplasm have been studied in relation to alterations in hormonal balance. The distribution of activity among separated fractions (mitochondrial, microsomal and supernatant fractions) has also been studied.

2. Adrenalectomy significantly lowered the ATP-ase activity of the whole cytoplasm and of the microsomal fraction. The activity in the microsomal fraction was likewise low after hypophysectomy, the effect of which could be reversed by administration of growth hormone.

3. Hypophysectomized rats showed a fall in the glucose 6-phosphatase activity of the whole cytoplasm and of the microsomal and supernatant fractions. The fall in cytoplasmic activity was partially reversed by administration of growth hormone.

4. Hypophysectomized rats (whether or not growth hormone had been given) and adrenalectomized rats showed a rise and a fall respectively in the xanthine oxidase activity both of the whole cytoplasm and of the supernatant fraction. No significant changes were found with hypothyroidism or hyperthyroidism.

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