Hormonal Influences on the Incorporation of Injected Precursors into the Protein and Ribonucleic Acid of Liver Cytoplasm

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When differential centrifuging was performed on rat-liver homogenates, prepared in 0.25M sucrose solution, evidence was obtained that certain cytoplasmic elements are selectively affected by changes in the hormonal status of the animal (Reid, 1955, 1956). For example, it appeared that the mitochondrial fraction is the site of the fall in liver ribonucleic acid (RNA) which is known to occur in untreated hypophysectomized rats and which, as Geschwind, Li & Evans (1950) have suggested, may reflect a reduction in protein synthesis.

Lee & Williams (1953) gave growth hormone to intact rats and observed an enhancement of the incorporation of labelled cystine, given by injection, into the protein of nuclear and mitochondrial fractions from liver. Otherwise there is no published evidence indicative of a relationship between hormonal status and the rate of protein synthesis, or of RNA synthesis, in the fractions obtained from liver by differential centrifuging.

Untreated hypophysectomized rats, hypophysectomized rats treated with growth hormone, and untreated adrenalectomized rats have now been compared with intact rats with respect to the incorporation, *in vivo*, of [1-¹⁴C]leucine into the protein of liver cytoplasmic fractions, and of [6-¹⁴C]orotic acid into the RNA of these fractions. Some of the observations have been briefly reported elsewhere (Reid & Stevens, 1956).

EXPERIMENTAL

Isotopically labelled compounds. DL-[1-¹⁴C]leucine was obtained from the Radiochemical Centre, Amersham.

[6-¹⁴C]orotic acid was prepared by the method of Heidelberger & Hurlbert (1950) from ethyl diethoxyacetate (Johnson & Cretcher, 1915) and ethyl [1-¹⁴C]acetate (Sakami, Evans & Gurin, 1947). Three millimoles (3.3 mc) of sodium [1-¹⁴C]acetate gave 253 mg. of recrystallized orotic acid monohydrate; equivalent weight 176 (theory 174); decomposition point 345°; yield from Ba¹⁴CO₃, 45%. The specific activity was 6.4 μC/mg.

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Animals and injections. The rats were albino males of the Institute colony, of age 10 weeks and body weight about 220 g. at the time of autopsy. Operations were performed at 7-8 weeks of age under Avertin (tribromoethanol) anaesthesia. The criterion for completeness of hypophysectomy, which was performed by the usual parapharyngeal approach, was diminution of testicular and adrenal weights to below one-third of those for equivalent intact animals. Bilateral adrenalectomy was performed by the usual dorsal approach; the rats were given 1% NaCl solution in place of drinking water, and were examined at autopsy for the possible presence of nodules of adrenal tissue.

The operated rats, together with intact control rats, were kept in individual cages on wire grids, and were maintained on the '20% protein diet' described by Elson (1952); the food intake of the intact and adrenalectomized rats was restricted to that of the hypophysectomized rats (usually 10 g./day, admixed with water). For 8 days before autopsy, some of the hypophysectomized rats received subcutaneous injections of growth hormone (Armour, Lot 527093B; thyrotrophin content less than 0.06 U.S.P. unit/mg.) at a dose level of 0.3 mg./day; these rats showed an average weight gain of 2.5 g./day. The average changes in weight for untreated hypophysectomized rats, for adrenalectomized rats and for intact rats were -1.0, +1.2 and +1.0 g./day respectively.

The rats were arranged in groups of four, with one or two intact control rats in each group. The rats in each group were killed on the same day, and the liver homogenates centrifuged simultaneously. To minimize the effect of any variations in environment or technique, the data for experimental rats were not directly averaged over all the groups, but were compared with those for corresponding control rats and the differences averaged. Altogether five untreated hypophysectomized rats, five growth-hormone-treated hypophysectomized rats and seven adrenalectomized rats were used in the study of leucine incorporation. The corresponding numbers for the study of orotic acid incorporation were four, four and six.

The isotopically labelled compounds were given by intraperitoneal injection usually in a volume of 0.4 ml. at about pH 8.5. The dose of DL-[1-¹⁴C]leucine was 2.05 mg., equivalent to 20 μC. The rats were killed 90 min. after the injection, at which time the radioactivity of the liver protein had barely attained a 'plateau' in intact rats. The dose of [6-¹⁴C]orotic acid was 1.25 mg., equivalent to 8 μC. The rats were killed 135 min. after the injection; at this time, as also found by Hurlbert & Potter (1952), the radioactivity of the liver RNA had not attained a plateau even in the nuclear fraction, the RNA of which shows the most rapid incorporation. Each rat received the same dose of leucine or orotic

acid irrespective of body weight, the dose being below the saturation level (cf. Keller, Zamecnik & Loftfield, 1954); the data of Table 1 include appropriate corrections for differences in body weight.

Isolation of tissue fractions. Each rat was finally anaesthetized with Nembutal (pentobarbital sodium), after an overnight fast. The liver was perfused *in situ* with cold 0.25 M sucrose solution, after (in some experiments) withdrawal of a blood sample from the heart. The radioactivity of the liver protein was no higher if perfusion was omitted, in contrast with the findings of Lee, Anderson, Miller & Williams (1951).

The liver was quickly excised and weighed. A weighed portion was homogenized in cold 0.25 M sucrose solution (12 ml./4 g. of liver) in a chilled homogenizer, with a Teflon pestle, as supplied by Arthur Thomas and Co., Philadelphia. The homogenates from the rats in each group were centrifuged for 10 min. at 800 g (3500 rev./min.). The debris thus obtained was rehomogenized in sucrose solution as before, and recentrifuged. These and subsequent centrifugings were performed at 0–3° in the high-speed head (Cat. no. 69404) of a refrigerated Medium centrifuge (Measuring and Scientific Equipment Ltd.); supernatant fluids were in general removed by pipette.

In the study of orotic acid incorporation, the debris (nuclear fraction) from the second centrifuging was re-suspended in sucrose, and a portion set aside for treatment with trichloroacetic acid solution as described below. In the study of leucine incorporation the nuclear fraction was discarded, since the radioactivity of contaminating cytoplasmic protein would have masked any change in that of nuclear protein.

The supernatant fluids from the two centrifugings were combined to give the cytoplasmic fraction, a portion of which was set aside for treatment with trichloroacetic acid solution. The remainder was centrifuged for 20 min. at 10 000 g (10 500 rev./min.). The material thus sedimented was separated into an underlying mitochondrial fraction and a fluffy layer, the latter being dislodged with 2 ml. of 0.25 M-sucrose solution by a swirling motion and removed by decantation. Because of the nature of this technique and of slight day-to-day variations in centrifuging speed, the yield of fluffy layer was rather variable.

The supernatant fluid was combined with the washings obtained on separately washing and recentrifuging the mitochondrial fraction and the fluffy layer. Further centrifuging, for 90 min. at 14 500 g (13 000 rev./min.), gave a microsomal fraction and a supernatant fraction. In some experiments a portion of the latter fraction was further centrifuged in a Spinco preparative ultracentrifuge, for 60 min. at 145 000 g (40 000 rev./min., Rotor 40), to sediment an ultracentrifugal fraction.

The various fractions thus obtained (including any blood samples) were treated with cold trichloroacetic acid solution (to 7%, w/v) and centrifuged after 15 min. The insoluble material was subjected to two thorough washings with cold 7% trichloroacetic acid solution, followed by treatment with hot ethanol and with hot ethanol-chloroform mixture (3:1, v/v). The products were finally dried overnight at 80°, weighed, and ground to a fine powder in a mortar.

The data for heart, kidneys and lungs were obtained with tissue which had been stored in the frozen state, and finally treated with trichloroacetic acid solution and lipid solvents as above.

Analyses. For the determination of radioactivity, each powdered sample was packed into the well (1 sq.cm.) of a polyethylene planchet, and counted in a thin-window counter. Duplicate counts agreed within 5%, the number of counts/min. being seldom less than five times background. Counting was performed at infinite thickness, except in the few cases where less than 30 mg. of material was available; in those cases an appropriate correction was applied.

L-Leucine and orotic acid are known to follow quite specific anabolic pathways, leading to protein and to nucleic acid respectively (Keller *et al.* 1954; Hurlbert & Potter, 1952). In the study of leucine incorporation, it was verified that the radioactivity of cytoplasmic fractions was undiminished after treatment with hot trichloroacetic acid solution (5%, 15 min. at 90°) to remove RNA, or with ninhydrin at pH 4.7 in the presence of unlabelled leucine as in the experiments of Winnick (1950). In the study of orotic acid incorporation, it was shown that cytoplasmic fractions had negligible radioactivity after treatment with hot trichloroacetic acid solution; moreover, when nuclear fractions were freed from RNA by the procedure of Schmidt & Thannhauser (1945), the DNA-containing residues gave very low counts which showed no consistent dependence on the hormonal status of the animal.

The RNA content of defatted fractions was measured by determining the extinction, at 260 m μ ., of acidified alkaline digests (cf. Schmidt & Thannhauser, 1945; Keller *et al.* 1954).

RESULTS

Values for radioactivity can usefully be expressed as percentage recovery, in protein or RNA, of injected isotope. As is evident from the upper part of Fig. 1, the amount of injected leucine recovered in the cytoplasmic protein of liver was diminished after hypophysectomy, especially if growth hormone had been given, and was also somewhat diminished after adrenalectomy. The several fractions isolated from cytoplasm by differential centrifuging shared proportionately in these changes. The difference between untreated and growth-hormone-treated hypophysectomized rats was almost significant ($P < 10\%$) in the cytoplasmic and microsomal fractions.

As is shown in the lower part of Fig. 1, the amount of orotic acid recovered in the crude nuclear material was rather variable; that in the cytoplasm as a whole tended to be low after hypophysectomy but was markedly raised after adrenalectomy. After hypophysectomy, the amounts incorporated into the RNA of the mitochondrial fraction, the fluffy layer and the microsomal fraction were decreased, in proportion to the amounts incorporated into these fractions in the intact controls. The supernatant and ultracentrifugal fractions did not, however, show a proportionate decrease. There was no indication of a difference in the incorporation of orotic acid between untreated and growth-hormone-treated hypophysectomized rats. Adrenalectomized rats had a diminished incorporation of

Fig. 1. Comparison of untreated hypophysectomized rats (bars with diagonal lines), of growth-hormone-treated hypophysectomized rats (bars with stipple), and of adrenalectomized rats (open bars) with intact rats, with respect to: (a) incorporation of DL-[1- 14 C]leucine into liver protein, as percentage recovery of injected isotope; (b) incorporation of [6- 14 C]orotic acid into liver RNA, as percentage recovery of injected isotope. The figures in parentheses are the mean values (percentage recovery) found for the intact rats. Vertical lines represent standard errors; where only two values were obtained they are indicated by large dots. Significant differences are denoted * ($P < 5\%$), ** ($P < 2.5\%$), *** ($P < 1\%$), **** ($P < 0.5\%$) or ***** ($P < 0.1\%$). The number of degrees of freedom (D.F.) is as given in Table 1, with the following exceptions for the orotic acid series; nuclear fraction, D.F. = 4 (hypophysectomized rats, either untreated or treated with growth hormone) or 6 (adrenalectomized rats); cytoplasmic fraction, D.F. = 4 (for each group); other fractions, D.F. = 7 (adrenalectomized rats only).

Table 1. *Specific radioactivity values for blood proteins and for fractions isolated from livers*

Animals were killed 90 min. or 135 min. after injection of radioactive leucine or orotic acid respectively. The standard error is given after each mean difference, except where less than four values were obtained. The number of degrees of freedom is given parenthetically. *P* denotes the probability that the difference could be due to chance.

Fraction	Control rats: mean value	Experimental rats: difference from corresponding control rats		
		Hypophysectomized	Hypophysectomized + growth hormone	Adrenalectomized
Incorporated leucine: $\mu\text{C/g.}$ of defatted protein fraction, after injection of $10\mu\text{C}$ of DL-leucine/100 g. body wt.				
Blood	0.46	-0.06 ± 0.053 (4)	-0.07 ± 0.043 (4)	-0.01 ± 0.019 (3)
Cytoplasmic (total)	1.45	-0.46 ± 0.135 (4; $P < 5\%$)	-0.50 ± 0.113 (4; $P < 2.5\%$)	-0.30 ± 0.115 (7; $P < 5\%$)
Mitochondrial (without fluffy layer)	1.04	-0.29 ± 0.044 (4; $P < 0.5\%$)	-0.41 ± 0.050 (4; $P < 0.5\%$)	-0.18 ± 0.071 (6; $P < 5\%$)
Fluffy layer	1.41	-0.37 ± 0.145 (3)	-0.17 ± 0.165 (4)	-0.02 ± 0.065 (6)
Microsomal	1.90	-0.43 ± 0.153 (4; $P < 5\%$)	-0.52 ± 0.083 (4; $P < 0.5\%$)	-0.02 ± 0.060 (6)
Supernatant	1.23	-0.42 ± 0.126 (4; $P < 5\%$)	-0.46 ± 0.111 (4; $P < 2.5\%$)	-0.17 ± 0.052 (6; $P < 2.5\%$)
Ultracentrifugal (from supernatant)	1.72	-0.58 (1)	-0.62 (1)	-0.10 ± 0.098 (3)
Incorporated orotic acid: $\mu\text{C/g.}$ of RNA, after injection of $4\mu\text{C}$ of orotic acid/100 g. body wt.				
Nuclear	13.0	$+0.25$ (2)	-0.95 (2)	-3.70 (2)
Mitochondrial (without fluffy layer)	2.16	-0.03 ± 0.250 (4)	-0.62 ± 0.308 (4)	$+0.78 \pm 0.322$ (6)
Fluffy layer	2.39	-0.54 ± 0.320 (4)	-0.48 ± 0.207 (4)	-0.17 ± 0.416 (6)
Microsomal	2.44	-0.70 ± 0.306 (4)	-0.92 ± 0.384 (4)	-0.88 ± 0.375 (6)
Supernatant	6.15	-0.51 ± 0.560 (4)	-0.89 ± 0.834 (4)	$+1.73 \pm 0.45$ (6; $P < 1\%$)
Ultracentrifugal from supernatant)	4.28	-0.92 ± 0.588 (4)	-1.05 ± 0.255 (4; $P < 2.5\%$)	$+0.39 \pm 0.238$ (4)

orotic acid into the microsomal fraction, but an increased incorporation into the supernatant fraction: this increase was not mainly located in the ultracentrifugal fraction.

In Table 1, the data are expressed in terms of specific activity rather than of recovery of injected isotope. The rough assumption may be made that the total leucine in the protein of the defatted fractions represents a constant percentage of the weight of the fractions.

It will be noted that the concentration of labelled leucine in the whole protein of blood was not significantly changed by hypophysectomy or by adrenalectomy, but that the concentration in the liver fractions showed the same trends as were observed with the values for percentage recovery. The specific activity values (not determined in every instance) for the RNA of liver fractions from rats given orotic acid also showed changes roughly parallel to those in percentage recovery; in particular it will be noted that the increased recovery of

isotope in the supernatant fraction after adrenalectomy was accompanied by a significant, although relatively smaller, rise in specific activity.

With tissues other than liver, the recovery of isotope was rather variable, probably because of the short interval between injection of isotope and autopsy (cf. Lee *et al.* 1951). Adrenalectomy did not significantly change the incorporation of leucine into the protein of heart, kidney or lung. There were no significant hormonal effects on the incorporation of orotic acid by the kidney or lung.

DISCUSSION

The finding that the incorporation of leucine into the protein of the various cytoplasmic elements from liver is diminished after adrenalectomy, and especially after hypophysectomy, is in agreement with observations made with whole liver by Lee & Williams (1952) and by Clark (1953). The observation that the incorporation of orotic acid into the

RNA of cytoplasmic elements is diminished after hypophysectomy is also in agreement with previous reports (Fraenkel-Conrat & Li, 1949; Logan, Heagy & Rossiter, 1955). The divergent effects of adrenalectomy on the supernatant fraction and on the microsomal (and possibly the nuclear) fraction, with respect to the incorporation of orotic acid, might account for the failure of Logan *et al.* (1955) to demonstrate any effect of adrenalectomy on the incorporation of labelled phosphate into the RNA of liver as a whole.

In contrast with the present findings, administration of growth hormone to hypophysectomized rats has been reported to cause an increased incorporation of labelled amino acids into liver protein (Lee & Williams, 1952; Gaebler, 1955), and also a 'suggestive increased turnover' of a crude nucleic acid fraction from liver after injection of labelled phosphate (Fraenkel-Conrat & Li, 1949). However, this nucleic acid fraction was compared with plasma inorganic phosphate, the specific activity of which is decreased when growth hormone is given (Geschwind, Li & Evans, 1951). Moreover, it is conceivable that the apparent effects of growth hormone in the experiments cited were due to increased food consumption. Preliminary experiments in this laboratory have indicated that when intact rats are given food *ad lib.*, rather than in restricted amount, as in the present study, greater amounts of leucine and of orotic acid are incorporated. The suggestion of Bartlett & Glynn (1950), that there is actually a translocation of protein from liver to muscle in hypophysectomized rats kept on a restricted food intake and given growth hormone, is compatible with the present findings.

Changes in incorporation, as now observed, can be regarded as indicative of changes in the rate of synthesis of protein or RNA only if neither the size of the liver pool, with respect to the immediate precursors of protein and of RNA, nor the extent of labelling of the pool, is markedly changed. There is published evidence (Awapara, Marvin & Wells, 1949; Kit & Awapara, 1954) to suggest that these conditions were probably satisfied in the study of leucine incorporation after adrenalectomy. There is indirect evidence, from experiments with labelled phosphate (Geschwind *et al.* 1951), that the changes in incorporation after hypophysectomy probably do not merely reflect changes in the pool. Since, however, the pool was not directly studied in the present experiments and since measurements were made after only a single interval of time, any interpretation of the hormonal effects in terms of changes in rates of synthesis must be regarded as purely tentative.

The data of Fig. 1 are compatible with the view that RNA, particularly that in the microsomal

fraction, plays a role in protein synthesis (cf. Keller *et al.* 1954; Brachet, 1955). This fraction showed a reduced incorporation both of leucine and of orotic acid after hypophysectomy or adrenalectomy. With the mitochondrial fraction and the fluffy layer the data give some indication of such a parallelism, but little elucidation of the previous observation (Reid, 1955, 1956) that the amount of RNA, in these fractions analysed conjointly, falls after hypophysectomy and is restored by growth hormone.

In the case of the supernatant fraction, however, hypophysectomy lowered the incorporation of leucine without proportionately lowering that of orotic acid. Moreover, adrenalectomy markedly increased the incorporation of orotic acid into the supernatant and ultracentrifugal fractions, without increasing the incorporation of leucine. A possible interpretation of these data is that the ultracentrifugal fraction, and particularly the whole supernatant fraction, may contain some RNA which has no connexion with protein synthesis. It should be pointed out that microsomal fractions as prepared in some studies of amino acid incorporation (Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin, 1955) would comprise both the microsomal fraction and the ultracentrifugal fraction as now prepared.

In the absence of data for the precursor pool, it is of course uncertain whether the actual rate of synthesis of the RNA in the supernatant fraction rose after adrenalectomy, particularly since this RNA might be derived in part from the nucleus (cf. Smellie, McIndoe, Logan, Davidson & Dawson, 1953) or might itself be a precursor of microsomal RNA (Barnum, Huseby & Vermund, 1953). Evidently, however, it is unlikely that the effect of adrenalectomy in increasing the amount of RNA in the supernatant fraction (Reid, 1955, 1956) is attributable merely to an enhanced loss of RNA from cytoplasmic particles during their isolation.

SUMMARY

1. Untreated hypophysectomized rats, hypophysectomized rats treated with growth hormone and adrenalectomized rats have been compared with intact rats, kept on the same food intake, with respect to the incorporation of DL-[1-¹⁴C]leucine into liver protein and of [6-¹⁴C]orotic acid into liver ribonucleic acid (RNA). The liver from each animal was separated by differential centrifuging into a nuclear fraction and a cytoplasmic fraction. The latter was centrifuged to give a mitochondrial fraction, a fluffy layer, a microsomal fraction, and a supernatant fraction which was, in some experiments, further centrifuged to sediment an ultracentrifugal fraction.

2. Hypophysectomized rats showed a decreased recovery of labelled leucine in cytoplasmic protein, each of the fractions sharing in this decrease. The recovery tended to be even lower after growth-hormone treatment in the case of the cytoplasm as a whole and of the microsomal fraction.

3. Adrenalectomized rats showed a decreased recovery of leucine in the protein of the cytoplasm as a whole and of the microsomal fraction, but the decrease was smaller than that observed after hypophysectomy.

4. Hypophysectomized rats showed a decreased recovery of labelled orotic acid in the RNA of the mitochondrial fraction, the fluffy layer, and the microsomal fraction. These effects of hypophysectomy were not significantly influenced by growth hormone.

5. Adrenalectomized rats showed a decreased recovery of orotic acid in the RNA of the microsomal fraction, but an increased recovery in that of the cytoplasm as a whole, of the supernatant fraction, and of the ultracentrifugal fraction.

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The Metabolism of the Monochloronitrobenzenes in the Rabbit

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The results of a detailed study of the metabolism of tetra- and penta-chloronitrobenzenes in the rabbit (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955) were of sufficient interest to justify an investigation of the metabolic fate of the complete series of chloromononitrobenzenes. One feature of the metabolism of these polychloronitrobenzenes which particularly attracted our attention

was that the mercapturic acids appeared to be formed by replacement of the nitro group by an acetylcysteyl group (acetylcysteylidenitration) and not by the usual process which is, in effect, an acetylcysteyldeprotonation and which occurs, for example, with naphthalene (Bourne & Young, 1934; Ing, Bourne & Young, 1934; Stekol, 1935), anthracene (Boyland & Levi, 1936) and the mono-