Biochem. J. (1961) **81**, 292

The Effect of Hypophysectomy and Growth-Hormone Treatment of the Rat on the Incorporation of Amino Acids into Isolated Liver Ribosomes

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(*Received* 20 *April* 1961)

Hypophysectomy of the rat results in changes in liver microsomes so that they are less able to incorporate amino acids into protein *in vitro* than are those from normal rat liver (Korner, 1959*a*). Treatment of the rats with ox growth hormone can reverse this change to some extent. Experiments on amino acid incorporation into liver-microsome protein in whole animals (Korner, 1959*b*) confirm the conclusion drawn from the experiments *in vitro* that hypophysectomy and growth hormone can exert an effect on microsomes which alters their ability to synthesize protein. The depressed rate of protein biosynthesis observed in livers of hypophysectomized animals and the stimulation of protein synthesis by treatment with growth hormone (see Ketterer, Randle & Young, 1957 for review) may be at least partly accounted for by such a change in the microsomes, since it is generally accepted (see Hoagland, 1960 for review) that much protein synthesis occurs in microsomes.

The sites of protein biosynthesis in the microsomes are the ribonucleoprotein microsomal particles (Littlefield, Keller, Gross & Zamecnik, 1955; Hoagland, 1960), and it has recently proved possible to prepare these microsomal particles or ribosomes (Roberts, 1958) from rat liver in a state in which they can incorporate amino acids into protein *in vitro* (Korner, 1959*c*, 1960, 1961; Takanami, 1960; Rendi & Hultin, 1960). This paper describes the investigation of the effects of hypophysectomy of the rat and of treatment of the rat with ox growth hormone, on the incorporation of amino acids into protein of isolated rat-liver ribosomes.

METHODS

Animals. Female albino rats were used. Some were hypophysectomized by the para-pharyngeal method of Ingle & Griffith (1942) and others were submitted to sham hypophysectomy. After recovery from the operation they were housed in individual cages and fed on the same ration of a powdered diet (diet 41, Bruce & Parkes, 1949) made into a thick pellet with water. The daily ration was the largest quantity of food which was entirely consumed by every rat used in the experiment (about 10 g. of diet/100 g. of rat/day), and the rats were maintained on this ration for at least 10 days before hormone treatment was begun.

Growth hormone. Ox growth hormone, prepared by the method of Wilhelmi, Fishman & Russell (1948) was dissolved in aq. 0·9% NaCl at pH 8·0 and diluted so that the dose could be given in 0·5 ml. of NaCl soln. by subcutaneous injection. Control rats were given 0·5 ml. of NaCl soln. in a similar manner.

Materials. Generally labelled L-leucine (L-[G-^{14}C]leucine), DL-[1-^{14}C]leucine, DL-[1-^{14}C]valine and hydrolysed algal protein labelled with ^{14}C were obtained from The Radiochemical Centre, Amersham, Bucks. The sodium salts of ATP and GTP were obtained from Sigma Chemical Co.

Preparation of ribosomes. The rats were starved overnight to deplete the liver of glycogen and were killed by decapitation. The livers were removed into ice-cold 0·44 M-sucrose and were cut up into small pieces with scissors. The liver was blotted and weighed by displacement in fresh, ice-cold 0·44 M-sucrose and the same weight of liver from control and treated groups of rats was homogenized in 5 vol. of 0·44 M-sucrose in a hand-operated homogenizer (Dounce, Witter, Monty, Pate & Cottone, 1955) kept cold in an ice bath. Care was taken to use the same number of strokes of the plunger in each case so that as far as possible the tissue from every group of rats was broken to the same extent. Nuclei, debris and mitochondria were removed by centrifuging at 15 000*g* for 10 min. at 0° and most of the supernatant fluid was removed. To it was added one-ninth of its volume of freshly made, ice-cold, 5% sodium deoxycholate in 0·03 M-tris hydrochloride buffer at pH 8·2, and the ribosomes were spun down from the dissolved lipoprotein by centrifuging at 105 000*g* for 2 hr. (cf. Korner, 1961). The supernatant fluid was removed, the pellets were rinsed in 0·03 M-tris at pH 7·5 containing 5 mM-$MgCl_2$, 0·05 M-NaCl and 0·08 M-KCl (medium *A*), and the insides of the tubes wiped with tissue. The ribosomes were washed to remove last traces of deoxycholate by gentle homogenization in medium *A* and they were centrifuged again at 105 000*g*. The ribosome pellets were suspended in medium *A* or in 0·045 M-tris at pH 7·5 containing 7·5 mM-$MgCl_2$, 75 mM-NaCl and 0·12 M-KCl (medium *B*) by gentle homogenization in a small hand-operated homogenizer and made up to a known volume with the same medium.

Preparation of microsomes, cell sap and pH 5 *enzyme.* Rat livers were homogenized as described above and centrifuged at 15 000*g* for 10 min., and the supernatant fluid was removed and centrifuged for 2 hr. at 105 000*g* at 0°. The cell sap was removed from the microsome pellet, which was suspended in medium *B*. A pH 5 enzyme preparation (Hoagland, Keller & Zamecnik, 1956) could be made from the cell sap by drop by drop addition of cold 0·1 N-acetic acid until the pH had fallen to 5, centrifuging at 0° and dissolving the precipitate in medium *A*. Any material that did not dissolve was removed by centrifuging, and the precipitation at pH 5 and subsequent solution in medium *A* was repeated.

Great care was taken to standardize the methods of preparing ribosomes, microsomes, cell sap and pH 5 enzyme so that they could be prepared in exactly the same way from livers of differently treated groups of rats.

Conditions of incubation. To 0·5 ml. of ribosome or microsome suspension in medium *B* was added 0·25 ml. of cell sap in 0·44 M-sucrose. In experiments in which pH 5 enzyme dissolved in 0·25 ml. of medium *A* was used instead of cell sap in sucrose, the ribosomes and microsomes were suspended in medium *A* and not medium *B* so that the concentration of salts in the final incubation mixture was the same in both types of incubation mixture. In either case 0·25 ml. of medium *A* containing the ATP, GTP, penicillin, chloramphenicol and radioactive amino acid and in which the pH had been readjusted to 7·5 was added. Thus in each case the final composition of the 1 ml. of reaction mixture was 0·03 M-tris buffer at pH 7·5, 5 mM-$MgCl_2$, 0·05 M-NaCl, 0·08 M-KCl, 5 mM-ATP, 0·6 mM-GTP, 100 units of penicillin, 40 μg. of chloramphenicol, 0·5 μC of radioactive amino acid, about 2 mg. of ribosome protein or 4 mg. of microsome protein and about 2·5 mg. of cell sap or pH 5 enzyme protein. When cell sap was used sucrose at a concentration of 0·11 M was also present. An ATP-generating system is not necessary (Korner, 1960, 1961). The mixture was incubated with shaking at 37° for 2 hr., the reaction stopped either by the addition of 0·5 N-$HClO_4$ or by addition of ice-cold 0·44 M-sucrose containing excess of unlabelled amino acid corresponding to the radioactive one used, and the microsomes or ribosomes were reseparated from the reaction mixture by centrifuging at 105 000*g* for 2 hr. The pellet was suspended in water, and the proteins of it and of the supernatant fluid were then precipitated with 0·5 N-$HClO_4$.

Preparation of radioactive protein for counting. The precipitated protein was washed free from radioactive amino acids, RNA and lipids were extracted and the protein was dried as described by Korner (1959*a*). The protein was then dissolved in 0·5 ml. of A.R. formic acid and then poured on to the centre of weighed stainless-steel planchets 3 cm. in diameter. The surface of these disks is divided into five compartments by four raised concentric rings so that fluid poured into the centre distributes itself evenly over the centre compartment and then overflows into the next compartment if its volume is too great for the centre. Thus each sample is distributed over the same area. The formic acid was evaporated off under an infrared lamp, the planchets were reweighed and the radioactivity of the protein was determined with an automatic Geiger–Müller gas-flow counter. Sufficient counts were recorded to reduce the standard error to 3 % or less and corrections were made for background counts. The results are expressed as the specific activity of the protein (counts/min./mg. of protein) or as counts/min./mg. of RNA.

Statistical treatment of results. For many of the experiments the livers of two or more rats were pooled to provide sufficient material. In each experiment replicate samples were incubated at each of the conditions studied, and the means of the results obtained with these samples were considered to constitute one experimental observation in order to overcome any variations in result that might arise by small differences in handling the material. Each experiment was repeated several times with other groups of rats kept under the same conditions, the mean of the results obtained in these separate experiments was calculated and Student's *t* test was applied to see if the difference between means was statistically significant.

Determination of ribonucleic acid. RNA was determined by the slight adaptation of the method of Scott, Fraccastoro & Taft (1956) that has been described by Korner (1959*a*).

Determination of nitrogen content. The nitrogen content of samples of the microsomes, ribosomes, cell sap, pH 5 enzyme and the residue after RNA determination was estimated by the micro-Kjeldahl technique. The value obtained was corrected for the nitrogen content of any tris or RNA present, and protein content calculated by multiplying the corrected nitrogen figure by 6·25.

RESULTS

Hypophysectomy and amino acid incorporation into ribosomes. Neither ribosomes nor cell sap, when incubated without the other in the usual incubation mixture, incorporate much amino acid into protein (Table 1) but considerable incorporation occurs when both are incubated together. Table 1 shows that ribosomes and cell sap prepared from livers of hypophysectomized rats incorporate about 43 % of the amount incorporated when the ribosomes and cell sap are prepared under identical conditions from normal rats. This difference is highly significant statistically ($P < 0{\cdot}001$). It is not the cell sap from livers of hypophysectomized rats that is deficient, however, for when this is

Table 1. *Incorporation of* DL-[1-^{14}C]*leucine into protein of ribosomes isolated from livers of normal and hypophysectomized rats*

The conditions are described in the Methods section. Results are the means of six experiments ± S.E.M.

Source of fractions incubated		Specific activity of ribosome protein (counts/min./mg. of protein)
Ribosomes	Cell sap	
Normal rats	—	19 ± 3·9
—	Normal rats	6 ± 1·4*
Normal rats	Normal rats	356 ± 21·9
Hypophysectomized rats	Hypophysectomized rats	152 ± 10·9
Hypophysectomized rats	Normal rats	169 ± 14·1
Normal rats	Hypophysectomized rats	329 ± 17·3

* Counts/min./mg. of cell-sap protein.

incubated with ribosomes from normal rat liver almost as much radioactivity is incorporated into their protein as when both parts of the system are prepared from normal rat liver. That hypophysectomy has affected the incorporating ability of the ribosomes is confirmed (Table 1) by the observation that ribosomes from hypophysectomized rats incorporate less than those from normal liver even if cell sap from normal rat liver is used in place of that from hypophysectomized rat liver.

Very similar results to those shown in Table 1 were obtained in experiments in which a pH 5 enzyme preparation was used in place of cell sap as a source of amino acid-activating enzymes (Hoagland *et al.* 1956) and soluble RNA (Hoagland, Zamecnik & Stephenson, 1957).

No consistent differences in RNA:protein ratio of ribosomes from normal and hypophysectomized rats was noted, consequently the differences in incorporation between them which have been found hold even if they are calculated as counts/min./mg. of RNA.

Growth-hormone treatment of rats and amino acid incorporation into ribosomes. Groups of hypophysectomized and normal rats were given various doses of ox growth hormone by subcutaneous injection for 5 days and the incorporation *in vitro* of amino acid into protein of ribosomes isolated from their livers was measured. The results (Table 2) show that treatment of hypophysectomized rats with growth hormone could restore the incorporation obtained almost to normal levels and that greater than normal levels of incorporation were found on treatment of normal rats with growth hormone. Experiments of the type depicted in Table 1 showed that most of the change brought about by treatment of rats with ox growth hormone could be accounted for by the increased ability of the ribosomes to incorporate amino acids into protein. Little change in the ability of the cell sap to prepare amino acids for incorporation was detected.

Effect of hypophysectomy on the stability of ribosomes. Korner (1961) has shown that ribosomes can be preincubated at 37° in medium *A* for about 1 hr. without substantial fall in their ability to incorporate amino acids into protein. It is possible that hypophysectomy of the rat may have altered the liver ribosomes in such a way that they are less stable than those from normal rat liver. The differences in amino acid incorporation found could then be explained by differences in the stability of the ribosomes. An examination of the increase in ribosome labelling with time of incubation (Fig. 1) does not, however, support this suggestion. Ribosomes from liver of hypophysectomized rats become radioactive more slowly than those of normal rats right from the beginning of incubation, and increases in the specific activity of ribosome protein in the preparation from hypo-

Table 2. *Incorporation of* DL-[1-^{14}C]*leucine into protein of ribosomes isolated from livers of normal and hypophysectomized rats injected with ox growth hormone*

	Growth hormone given each day for 5 days (mg.)	Counts/min./mg. of ribosome protein
Normal rats	0	348±29
	0·01	391±22
	0·25	407±19
	1·0	401±31
Hypophysectomized rats	0	172±22
	0·01	249±14
	0·25	283±17
	1·0	298±24

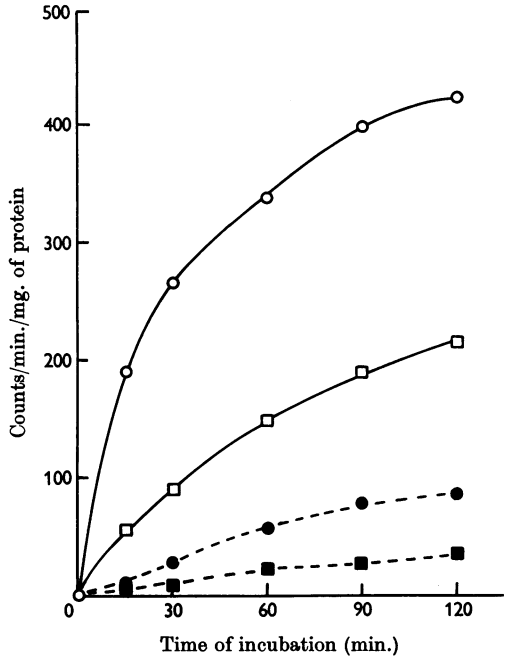

Fig. 1. Time-course of incorporation of L-[G-^{14}C]leucine into protein of ribosomes and cell sap isolated from livers of normal and hypophysectomized rats. Incubation of ribosomes and cell sap from normal rat liver and from hypophysectomized rat liver was carried out under conditions described in the text. At the end of incubation ribosomes and cell sap were separated from each other as described in the Methods section and the radioactivity of the proteins of each was assessed. ○, Ribosomes from normal rats; □, ribosomes from hypophysectomized rats; ●, cell sap from normal rats; ■, cell sap from hypophysectomized rats.

physectomized rats keep more or less in step with increases in specific activity obtained with ribosomes from normal rat liver.

The results in Table 3 offer support for the contention that no difference is apparent in the stability of ribosomes as a result of hypophysectomy or treatment with growth hormone, for little difference in the ability of ribosomes from the differently treated groups of rats to withstand preincubation was detected.

Transfer of protein to supernatant fluid. When ribosomes are incubated, radioactive protein appears in the supernatant fluid, and it has been suggested, but not proved, that this occurs by transfer of labelled protein from ribosomes to supernatant rather than by breakdown of ribosomes (Korner, 1961). Similar percentages of the total counts in the incubation mixture appeared in non-ribosomal protein at various times during an experiment whether the ribosomes came from livers of normal rats or from livers of hypophysectomized rats. This conclusion is illustrated in Fig. 1, which shows that the relationship of specific activity of the cell-sap protein to that of the ribosome protein at different times of incubation is similar in the systems prepared from normal and hypophysectomized rat liver.

Table 3. *Effect of preincubation of ribosomes from livers of normal and hypophysectomized rats and from rats treated with growth hormone on their subsequent ability to incorporate radioactive amino acids into protein*

Incorporation at zero time was taken as 100.

Time of preincubation (min.)	Ribosomes from: Normal rats	Ribosomes from: Hypophysectomized rats
0	100	100
15	96	93
30	90	91
60	81	78

Magnesium and adenosine triphosphate concentration on amino acid incorporation into ribosomes from normal and hypophysectomized rat liver. The concentration of magnesium ions and of ATP and the relative proportions of them are critical variables in controlling the amount of amino acid which is incorporated into ribosomes (Korner, 1961). Incorporation of amino acid into protein of ribosomes from hypophysectomized rats showed the same behaviour towards these variables as the ribosomes from normal rat liver. The best incorporation was seen at 0·01 M-ATP and 0·01 M-$MgCl_2$ in both cases (cf. Korner, 1961). Many other concentrations of magnesium and ATP were tested and these caused large differences in the incorporation obtained, but in each case the incorporation of amino acids into ribosomes from normal rat liver was always greater than that into ribosomes from hypophysectomized rat liver.

Comparison of the effects of hypophysectomy on microsomes and on ribosomes. A group of rats were hypophysectomized and they, and some sham-operated control rats, were treated with various doses of ox growth hormone. Homogenates of liver of each group of rats were made in the usual way, and both microsomes and ribosomes were prepared from the same homogenate. The extent of incorporation of amino acids into protein in the microsomes and ribosomes was compared under standard conditions with the same pH 5 enzyme preparation in each case. Table 4 shows that, when compared with untreated control rats, hypophysectomy or growth-hormone treatment produced broadly similar percentage changes in specific activity of protein of ribosomes and of microsomes.

DISCUSSION

The experiments reported in this paper support the idea that hypophysectomy of the rat changes the liver ribosomes in a way that makes them less capable of synthesizing protein than those of normal rat liver and that treatment of rats with ox growth

Table 4. *Incorporation of* DL-[^{14}C]*valine into protein of ribosomes and microsomes from livers of normal and hypophysectomized rats and rats treated with ox growth hormone*

	Growth hormone given each day for 5 days (mg.)	Counts/min./mg. of microsome protein	Change compared with untreated normal rats (%)	Counts/min./mg. of ribosome protein	Change compared with untreated normal rats (%)
Normal rats	0	168	—	404	—
	0·01	198	17·9	482	19·3
	1·0	217	29·2	507	25·3
Hypophysectomized rats	0	70	−58·4	157	−61·0
	0·01	119	−29·2	317	−21·5
	1·0	135	−19·7	351	−13·1

hormone causes changes in ribosomes which enhance their protein-synthesizing ability. Little change was detected in the activity of the amino acid-activating enzymes (Hoagland *et al.* 1956) or in the ability of the soluble RNA (Hoagland *et al.* 1957) to form a complex with amino acids and to transfer it to ribosomes.

The possibility that the changes in incorporation of amino acids into protein resulting from hypophysectomy were brought about by altered stability of the ribosomes or by changes in their requirements for incorporation was ruled out by some of the experiments described (Fig. 1; Table 3). Support for the idea that growth hormone affects ribosomes *in vivo* in a way that enables them to incorporate more amino acids in the *in vitro* system is provided by these experiments and this suggestion is reinforced by the results of experiments with whole animals (Korner, 1959*b*).

The changes that are known to occur in the amino acid-incorporating ability of microsomes as a result of hypophysectomy of rats or treatment of rats with growth hormone (Korner, 1959*a*) can be explained by changes in the ribosome part of them, for the percentage change in incorporation after hypophysectomy and growth-hormone administration was the same in ribosomes as in microsomes.

The change in ribosomes brought about by growth-hormone treatment or hypophysectomy of the rat does not appear to alter the ability of ribosomes to release labelled protein to the supernatant fluid (Fig. 1), so the change must have occurred in the ability of the ribosome to accept activated amino acids and to link them in polypeptide chains.

Little or no difference in RNA:protein ratio was detected between ribosomes from livers of normal and hypophysectomized rats. Perhaps there is less non-active RNA in ribosomes from liver of rats that have circulating growth hormone or there may be less inactive ribosomes. It has been suggested for at least one system (Tissière, Schlessinger & Gros, 1960) that only a very small proportion of the total ribosomes of the cell are able to incorporate amino acids into protein; it is possible that the number that are active is controlled in some way by growth hormone.

The results reported in this paper offer an at least partial explanation of the known effects of hypophysectomy and of growth-hormone treatment on body weight, carcass protein, nitrogen retention and protein biosynthesis (Lee & Schaffer, 1934; Lee & Ayres, 1936; Simpson, Evans & Li, 1949; Russell, 1951, 1955; Ketterer *et al.* 1957), in terms of hormonally-induced changes of the templates on which protein synthesis occurs (Littlefield *et al.* 1955; Hoagland, 1960).

SUMMARY

1. Hypophysectomy of the rat reduces, and treatment of normal and of hypophysectomized rats with ox growth hormone increases, the incorporation of radioactive amino acids into protein of isolated liver ribosomes.

2. The ribosomes from hypophysectomized rat liver were no less stable than those from normal rat liver, nor did their requirements for incorporation differ significantly from those of normal rat liver.

3. A similar proportion of labelled protein was transferred to the supernatant fluid from ribosomes of liver of hypophysectomized rats as from those of normal rats.

4. The effects of hypophysectomy of the rat and treatment with growth hormone on microsomes that have been previously demonstrated (Korner, 1959*b*) can be explained in terms of the hormonal effects on the ribosome part of the microsomes.

5. It is concluded that the effects of growth hormone on protein biosynthesis in rats can be explained at least partly in terms of hormonally induced changes of the ribosomes in which protein synthesis takes place.

I wish to thank the Medical Research Council for an expenses grant, the British Drug Houses Ltd. for a grant for technical assistance, Mrs B. Jones, for highly skilled assistance, Miss M. B. Thomas, who performed the hypophysectomies, and Mr B. Slater, who prepared the ox growth hormone. It is a pleasure to thank Professor F. G. Young, F.R.S., for his interest and encouragement.

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The Inhibition of Acetylcholine Synthesis in Brain by a Hemicholinium

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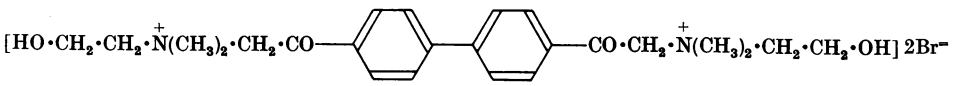
(*Received* 30 *March* 1961)

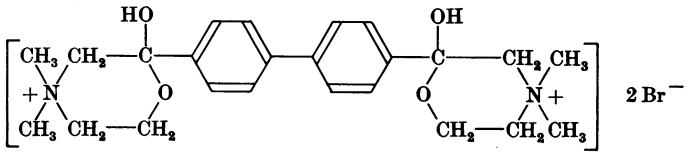
Long & Schueler (1954) described a series of bisquaternary ammonium compounds based on αα′-dibromo-4:4-bisacetophenone including one, obtained by treating the dibromo compound with dimethylaminoethanol, which was formulated as in Fig. 1*a*. As it was extremely toxic when injected into animals but possessed only weak anticholinesterase activity, and as the infrared spectrum showed strong bands due to ether groups, Schueler (1955) suggested the hemiacetal formulation (Fig. 1*b*) and described the compound as a 'hemicholinium'. The compound of Fig. 1 is usually referred to as HC3.

Long & Schueler found that, when injected into animals, HC3 paralysed the respiration; the paralysis developed after some delay and was reversed by injecting choline or anticholinesterases. MacIntosh, Birks & Sastry (1956) found that low concentrations of HC3 (0·01–0·1 mM) inhibited the synthesis of acetylcholine in the perfused superior cervical ganglion of the cat and in minced brain of the mouse; the inhibition was reversed by adding choline in similar concentrations. MacIntosh *et al.* (1956) therefore suggested that HC3 somehow inhibited the formation of acetylcholine. However, choline acetylase extracted from acetone-dried powder of brain was hardly inhibited at all by HC3, even when the concentration of HC3 was one thousand times that of choline. This made it unlikely that the inhibition of acetylcholine formation thought to occur *in vivo* was due to a direct effect of HC3 on choline acetylase.

The possibility remained, however, that the absence of inhibition *in vitro* was either because the enzyme was changed during its extraction or because HC3 competed with choline for access to the enzyme in the cell. These alternatives had to be examined because in the brain 75 % or more of the choline acetylase is associated with intracellular particulate fractions that are fully active only after



(*a*)



(*b*)

Fig. 1