The Effect of Hypophysectomy of the Rat and of Treatment with Growth Hormone on the Incorporation of Amino Acids into Liver Proteins in a Cell-free System

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Removal of the pituitary gland from an animal results in inhibition of growth, and treatment with growth hormone can, in suitable circumstances, cause a hypophysectomized animal to increase in body weight (see Ketterer, Randle & Young, 1957). However growth is defined (Weiss, 1955), protein synthesis must be intimately concerned in the process, and the implication of the many experiments with hypophysectomized animals is that certain of the secretions of the pituitary gland, especially growth hormone, influence protein synthesis. The liver, among other organs of the rat, decreases in size and protein content as a result of hypophysectomy, and this fall can be prevented to a large extent by treatment with growth hormone (Lee & Schaffer, 1934; Simpson, Evans & Li, 1949). Siekevitz (1952) and Zamecnik & Keller (1954) have shown that rat liver can be used as the source of a cell-free system which will incorporate amino acids into protein *in vitro*. Evidence is accumulating which suggests that many of the reactions studied with this system are concerned in protein biosynthesis *in vivo* and, indeed, that protein synthesis may be occurring to a small extent in this cell-free system. This paper describes the investigation, in the rat, of the effect of hypophysectomy, and of treatment with growth hormone, on the incorporation of amino acids into protein *in vitro* in the cell-free system of Zamecnik & Keller (1954).

METHODS

Animals. Female albino rats were used and in some experiments they were allowed to eat freely of a mixed diet. Because the amount of food eaten influences the rate of protein synthesis, in most experiments the rats were housed in individual cages and each given the same ration of a powdered diet (Diet 41, Bruce & Parkes, 1949) made up into a thick pellet with water. The daily ration, established by trial and error, was the largest quantity of food which would be entirely consumed by every rat of an experimental group. The rats were kept on this ration (about 10 g. of diet 41/100 g. of rat/day) for at least 10 days before treatment with hormones was begun. Hypophysectomy, by the para-pharyngeal method (Ingle & Griffith, 1942), was carried out at least three weeks before the animals were used in an experiment, completeness of removal of the pituitary being checked by observation at

autopsy. A sham operation was carried out on the control rats used in some of the experiments.

Growth hormone. Ox growth hormone was used and was prepared by the method of Wilhelmi, Fishman & Russell (1948). It was dissolved in aq. 0.9% NaCl at pH 8.0 and the appropriate dose was always injected subcutaneously in 0.5 ml. of NaCl solution. Control rats were similarly given 0.5 ml. of aq. 0.9% NaCl.

Radioactive compounds. Generally (G) labelled L-[G-¹⁴C]-leucine and DL-[1-¹⁴C]leucine were used in most of the experiments, but some results were obtained with DL-[1-¹⁴C]valine, DL-[β-¹⁴C]tryptophan and hydrolysed algal protein labelled with ¹⁴C. These radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks.

Materials. The sodium salts of adenosine triphosphate (ATP) and of guanosine triphosphate were obtained from the Sigma Chemical Co. The sodium salt of creatine phosphate was prepared by the method of Ennor & Stocken (1948) and creatine phosphokinase was prepared by the method of Kuby, Noda & Lardy (1954).

Preparation of the cell-free system. The method was that of Zamecnik & Keller (1954). Rats were killed by decapitation and were bled. The liver was rapidly removed and cut up with scissors into an ice-cold homogenizing medium consisting of 0.03 M-KHCO₃, 0.025 M-KCl, 0.35 M-sucrose and 0.01 M-MgCl₂ in 0.02 M-KH₂PO₄-K₂HPO₄ buffer at pH 7.5. The 0.01 M-MgCl₂ was the last solution added during the preparation of the medium, which was gassed for 1 hr. with O₂ + CO₂ (95:5) before use. The pH was measured with a glass electrode standardized with a standard buffer solution made up, in accordance with the instructions of the manufacturers, from Burroughs Wellcome and Co.'s N.B.S. buffer solution tablets. The pH of the standard solution was 6.9 at 25°. A portion of the liver was blotted, weighed by displacement in ice-cold medium and homogenized in 3 vol. of the medium in a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept cold in an ice bath. Only three strokes (up and down) were used since severe damage to the tissues diminishes its capacity to incorporate amino acids *in vitro* (Zamecnik & Keller, 1954).

The resulting brei was spun at 15 000 g for 10 min. at 0° in a refrigerated centrifuge to remove unbroken cells, cell debris, nuclei and mitochondria, and the supernatant fluid, which is referred to as the cell-free system and which contains the microsomes and the soluble fraction of the cell, was used as quickly as possible after preparation.

Separation of microsomes and soluble fraction. The supernatant fluid obtained by centrifuging at 15 000 g could be separated into microsomes and the soluble fraction of the

cell by spinning at 105 000 *g* in a Spinco Model L preparative ultracentrifuge for 1 hr. at 0°. The microsome pellet was rinsed in the medium, suspended by gentle homogenization in fresh medium and made up to a known volume. Great care was taken to standardize rigidly the method of preparation of the cell-free system, of the microsomes and of the soluble fraction, so that they could be prepared in exactly the same way from livers of differently treated groups of rats.

Conditions of incubation. Samples of the cell-free system, or of the microsomal and soluble fractions containing the required amount of protein, were mixed with ATP solution containing equimolar MgCl_2 , and with creatine phosphate solution, so that the final concentration of ATP and creatine phosphate were 6 and 23 mM respectively. The radioactive amino acids, dissolved in the ATP medium, were added to obtain a final concentration of 0.13 mM (about 0.4 $\mu\text{C}/\text{ml}$. of final vol.). The total volume of the mixture was adjusted to 1 ml. with the buffered salt medium. The pH of all solutions was adjusted to 7.5 with 0.1N-KOH before they were added to the mixture. The reaction was stopped by the addition of cold 0.5N- HClO_4 , or, if the microsomes were to be removed from the soluble fraction, the contents of the tubes were poured into cooled Spinco centrifuge tubes containing ice-cold 0.25M-sucrose together with an excess of unlabelled amino acid, and the tubes centrifuged at 105 000 *g* for 1 hr. The proteins of the soluble fraction and of the resuspended microsomes were then precipitated with cold 0.5N- HClO_4 .

Preparation of a freeze-dried system. Recently Sachs (1957) has found that the cell-free system prepared from rat liver may be freeze-dried and stored at -20° for some months without loss of the ability to incorporate amino acids into proteins *in vitro*. Some of the experiments described in this paper were carried out with material prepared and kept in such a fashion.

The whole liver was excised as before, cut up into ice-cold 0.25M-sucrose, and homogenized in the way described above. The brei was then centrifuged at 15 000 *g* for 10 min. at 0°, and the supernatant fluid shell-frozen in acetone- CO_2 , freeze-dried and stored at -15°. For an experiment this material was dissolved, in centrifuge tubes, in a medium consisting of 0.03M-2-amino-2-hydroxy-methylpropane-1:3-diol hydrochloride (tris) buffer at pH 7.5 containing 0.08M-KCl, 0.05M-NaCl, 5 mM- MgCl_2 and 2.5 mM-glutathione (GSH). The pH was measured with a glass electrode standardized against a standard buffer solution. Microsomes and soluble fraction were separated from the freeze-dried material in the same way as with fresh tissue (see above) and the microsomes suspended by gentle homogenization in the tris medium. Material prepared in this way was shaken at 37° for 30 min. in the presence of mM-ATP, 20 mM-creatine phosphate, 0.6 mM-guanosine triphosphate, 0.03 mg. of creatine phosphokinase and 0.13 mM radioactive amino acid (about 0.4 μC) in a total volume of 1 ml. The reaction was stopped by the addition of either cold 0.5N- HClO_4 or ice-cold sucrose containing excess of unlabelled amino acid, and the microsomes were removed as described above.

Preparation of radioactive proteins. The precipitated protein was washed three times with cold 0.5N- HClO_4 containing excess of unlabelled amino acid to remove radioactive amino acid. Ribonucleic acid (RNA) was removed by extraction with N-NaOH at room temperature

for 1 hr., after which the mixture was acidified with 6N-HCl (Scott, Fraccastoro & Taft, 1956). The residual material was boiled with 0.5N- HClO_4 and extracted twice at room temperature with a chloroform-ethanol-ether mixture in the ratios of 1:2:2 (by vol.), in order to remove lipids, and the powder was then dried with ether.

Weighed aluminium planchets, 5 cm.² in area, were screwed into the caps of 4 oz. screw-capped jars. The jars had been cut off at the bottom of the neck so as to leave a short column of glass above the cap. A slurry of the dry protein powder was made with a mixture of ether and light petroleum (1:1, v/v) and this slurry was poured on to the planchet in its bottle top and the solvent allowed to evaporate. In this way 1-15 mg. of protein powder can be evenly plated onto the planchet. The planchets were then reweighed, and the radioactivity of the protein was estimated by counting with a Geiger-Müller end-window counter (1.5-2.5 mg./cm.²). Sufficient counts were recorded to reduce the standard error to 3% or less, and corrections were made for the background counts and for self-absorption. The results are given as the specific activity of the protein (counts/min./mg. of protein).

Statistical treatment of results. In those experiments where enough material could not be obtained from one liver, the livers of several rats which had been treated in the same way were pooled and the cell-free system was prepared from the pooled material. In every experiment replicate samples from the liver of one rat, or pooled livers of a group of rats, were incubated under each of the conditions studied and the mean value of the specific activities of their protein was calculated and considered to constitute one experimental observation. Each experiment was repeated several times with the livers from rats kept under identical conditions, and the mean of the experimental observations, together with the standard error of their means (s.e.m.), calculated. Student's *t* test was applied to ascertain whether the difference between means was statistically significant, and values of Fisher's *P* are given when a difference between mean values is considered to be statistically significant.

Determination of ribonucleic acid. Ribonucleic acid (RNA) was determined in samples of microsomal and soluble fractions by a slight adaptation of the method of Scott *et al.* (1956) in which the cold 0.5N- HClO_4 ppt. of the sample was washed with cold 0.5N- HClO_4 and with cold ether-ethanol (1:1, v/v) and then extracted twice for 1 hr. at room temp. with N-NaOH. The mixture was then acidified with 6N-HCl. The RNA content of the combined alkaline extracts was determined from the extinction at 260 μm in the Beckman spectrophotometer by using an extinction coefficient of 34.2/mg./ml./cm. (Scott *et al.* 1956).

Determination of protein content. The nitrogen content of samples of the fractions incubated was estimated by the micro-Kjeldahl technique. Corrections were applied for the nitrogen content of any tris buffer present and of the RNA in the samples (by using 14% as the nitrogen content of RNA) and the protein content of the samples was calculated from this corrected nitrogen-content figure by using the factor 6.25. These data were used in some experiments to calculate the specific activity of the microsomal protein by the expression $SPX/100M$, where *S* is the specific activity of the total protein isolated, *P* is the weight (mg.) of protein incubated, *M* is the weight (mg.) of microsomal protein incubated and *X* is the percentage of the total

radioactivity in the protein of the sample which is found to be present in the microsomal protein fraction. X was determined by removing the microsomes from the soluble fraction in two or three of the samples at the end of each of the incubation periods, and determining the specific activity of the protein of each fraction. This observed value of X was then used to calculate the specific activity of the microsomal protein in the rest of the samples. Because the number of samples was very large any attempt to divide them all into microsomes and soluble fraction at the end of the experiment was precluded, and in any case the error introduced by assuming that the value of X could be applied to all the samples of one type in an experiment was found by trial to be very small. If the results presented have been calculated by use of this formula a note to this effect appears in the legend to the Table or Figure concerned.

Determination of glycogen. The glycogen content of the liver was determined by a modification of the method of Good, Kramer & Somogyi (1933).

RESULTS

Incorporation of amino acid into protein in the cell-free system. Fig. 1 shows the results of a typical experiment in which the cell-free system (i.e. the

Fig. 1. Specific activities of the proteins of cell-free systems after incubation at 37° for various times with DL-[1-¹⁴C]leucine. Protein (8–10 mg.) from the livers of normal (○), hypophysectomized (△) or hypophysectomized rats treated with 0.2 mg. of growth hormone/day for 5 days (●) were incubated in 0.02 M-KH₂PO₄-K₂HPO₄ buffer (pH 7.5), containing 0.03 M-KHCO₃, 0.025 M-KCl, 0.35 M-sucrose and 0.01 M-MgCl₂, together with 6 mM-ATP, 23 mM-creatine phosphate and 0.13 mM-DL-[1-¹⁴C]leucine (0.4 μC) in a total vol. of 1 ml.

whole supernatant fluid after centrifuging at 15 000 g) is incubated with DL-[1-¹⁴C]leucine under the conditions described. The specific activities of the proteins of the cell-free system, prepared from the livers of normal rats, are compared with the specific activities of the proteins of the cell-free system, prepared in exactly the same way from the livers of hypophysectomized rats, after various periods of incubation under identical conditions.

It can be seen that amino acid is rapidly incorporated into protein and that a plateau level of specific activity is reached in about 20 min. in both instances, but that much less amino acid is incorporated into the proteins of the preparation from the livers of hypophysectomized rats than into the proteins of the liver preparation from normal rats. Many experiments of this type have been carried out; whenever the conditions of the experiment led to the optimum incorporation of amino acid by the proteins of the cell-free preparation from normal rats, the specific activity of the protein found in the companion preparations from hypophysectomized rats was about 40% or less of the value found for preparations from normal animals. In earlier experiments, when a search was being made for the optimum conditions of preparation and incubation of the cell-free system, the conditions differed somewhat from those described above and the specific activity of the proteins of the cell-free system from normal rat liver was less than the values indicated in Fig. 1. But in all such instances the amino acid incorporated into the protein from hypophysectomized animals was less than that with preparations from normal rats, ranging from 20 to 70% of that found with the protein from normal rats (Korner, 1958).

Treatment of the hypophysectomized rats with ox growth hormone made possible the preparation of a cell-free system which incorporated more amino acid into protein *in vitro* than did the system obtained from the untreated hypophysectomized rats. Fig. 1 shows the incorporation of DL-[1-¹⁴C]leucine into protein of liver preparations from hypophysectomized rats given 0.2 mg. of growth hormone/day for 5 days. In no instance was treatment with ox growth hormone alone successful in restoring the extent of incorporation of amino acid by the cell-free preparation from hypophysectomized rats to that of preparations from normal rats.

When microsomes and soluble fraction are prepared from the cell-free system by centrifuging at 105 000 g, the amino acid-incorporating system has been separated into two parts, neither of which alone incorporates amino acids to any great extent when shaken at 37° in the presence of ATP, creatine phosphate, creatine phosphokinase, guanosine triphosphate and radioactive amino acids.

Table 1. *Incorporation in a cell-free system of DL-[1-¹⁴C]leucine into microsomal protein prepared from livers of normal and hypophysectomized rats*

3 mg. of microsomal protein and 4 mg. of soluble-fraction protein were incubated for 30 min. at 37° in 0.03 M-tris buffer (pH 7.5) containing 0.08 M-KCl, 0.05 M-NaCl, 5 mM-MgCl₂, 2.5 mM-GSH, mM-ATP, 0.02 M-creatine phosphate, 0.6 mM-guanosine triphosphate, 0.03 mg. creatine phosphokinase and 0.13 mM-DL-[1-¹⁴C]leucine (0.4 μC) in a total vol. of 1 ml. The specific activity of the microsomal protein was calculated from the formula given in the Methods section.

Source of protein fractions incubated		Specific activity of microsomal protein (mean ± s.e.m.) (counts/min./mg.)
Microsomes	Soluble fraction	
Normal rats	—	48 ± 7.3
—	Normal rats	4 ± 1.3*
Normal rats	Normal rats	187 ± 10.1
Hypophysectomized rats	Hypophysectomized rats	71 ± 6.0
Hypophysectomized rats	Normal rats	78 ± 5.2
Normal rats	Hypophysectomized rats	178 ± 9.2

* Counts/min./mg. of soluble-fraction protein.

More amino acid is incorporated into microsomal protein incubated alone than into soluble fraction protein incubated alone under these conditions, presumably because the microsomal pellet traps some of the soluble fraction so that some amino acid can be activated by the amino acid-activating enzymes present in the soluble fraction of the cell (Hoagland, Keller & Zamecnik, 1956), and some amino acid-soluble ribonucleic acid intermediate (Hoagland, Zamecnik & Stephenson, 1957) can be formed (see Discussion). Incorporation of amino acid to the same extent as in the unseparated system does occur when both microsomes and soluble fraction are incubated together, and it can be seen from Table 1 that when microsomes from normal rats were incubated with soluble fraction from normal rats the mean specific activity of the microsomal protein was 187 counts/min./mg. The mean specific activity of the protein of microsomes from hypophysectomized rats, after incubation with soluble fraction from hypophysectomized rats, was 38% of that of the protein from normal rats. The difference between these means is statistically highly significant ($P < 0.001$). When microsomes from hypophysectomized rats were incubated with soluble fraction from normal rats the specific activity was almost the same as when microsomes and soluble fraction, both from hypophysectomized rats, were incubated together, showing that alterations in the soluble fraction from livers of hypophysectomized rats were not responsible for the diminution in incorporation of amino acid by the preparation from hypophysectomized rats. Again, incubation of microsomes from normal rats with soluble fraction from hypophysectomized rats resulted in almost the same specific activity as when microsomes and soluble fraction from normal rats were incubated together. In other words, the soluble fraction from the livers of hypophysectomized rats is just as able as the soluble fraction

Table 2. *Incorporation of DL-[1-¹⁴C]leucine into microsomal protein in cell-free systems prepared from livers of two normal rats*

3 mg. of microsomal protein and 4 mg. of soluble-fraction protein were incubated with 0.13 mM-DL-[1-¹⁴C]leucine (0.4 μC) at 37° for 30 min. Conditions of incubation are given in the legend to Fig. 1.

Source of the protein fractions incubated		Specific activity of microsomal protein (counts/min./mg.)
Microsomes	Soluble fraction	
Rat A	Rat A	162
Rat B	Rat B	154
Rat A	Rat B	152
Rat B	Rat A	149

from the livers of normal rats to prepare amino acids for incorporation into protein, at least to the extent required by the microsomes from normal rats. The diminished incorporation of amino acid into protein found in the complete preparation from the livers of hypophysectomized rats must be caused by some alteration in the microsomes. Any alteration of the ability of the soluble RNA to form a complex with the amino acid and to transfer it to the microsomal particle, which may result from hypophysectomy, cannot be the limiting factor during incorporation of amino acid into protein in this system.

Similar results to those described in Table 1, which were carried out with freeze-dried material, were obtained in experiments with material which had not been freeze-dried.

Table 2 gives the results of an experiment which was designed as a check of the accuracy of the method. From two samples of liver (A and B), one from each of two normal rats, the microsomes and soluble fraction were prepared in the way described. It can be seen from the table that the specific

Table 3. Specific activity of microsomal protein from rat liver after 30 min. incubation in the cell-free system with L-[G-¹⁴C]leucine after various preincubation procedures

The conditions of incubation are as in Fig. 1. Procedure 1: no preincubation; procedure 2: preincubation of whole homogenate for 15 min. at 37°; procedure 3: preincubation of microsomes and soluble fraction together for 15 min. at 37°; procedure 4: preincubation of microsomes and soluble fraction separately for 15 min. at 37°. The counts/min./mg. of microsomal protein are calculated from the formula given under Methods.

Treatment of microsomes ...	Counts/min./mg. of microsomal protein			
	Procedure 1	Procedure 2	Procedure 3	Procedure 4
Treatment of soluble fraction				
Procedure 1	165	158	136	72
Procedure 2	10	12	—	—
Procedure 3	87	—	58	—
Procedure 4	132	—	—	67

activity of the microsomal protein from preparation A and from preparation B, after incubation under the usual conditions, was approximately the same whether the microsomes from A were incubated with the soluble fraction from A or from B or whether the microsomes from B were incubated with soluble fraction from A or B.

Possibility of degradation of the microsomes in vitro. It seemed possible that the microsomes of hypophysectomized rats are initially just as able as those from normal rats to incorporate amino acids into protein *in vitro*, but that this ability falls off rapidly during the experiment and results in a difference in the amount of amino acid incorporated by the two types of microsome over a period of time. The possible rise in the rate of breakdown could be caused by a greater activity of proteolytic or of ribonuclease enzymes in preparations from hypophysectomized rats than in those from the normal rat, or by a greater sensitivity to the activity of such degradative enzymes of the microsomes from hypophysectomized rats than of those from normal rats. Roth (1954) has indeed suggested that the ribonuclease (RNAase) enzymes of rat liver may be concerned in protein synthesis. An experiment was carried out to test the possibility that the difference found between the microsomes of normal and hypophysectomized rat liver might result from a greater breakdown of the microsomes from hypophysectomized rats' liver under the conditions of these experiments.

Homogenates from the livers of normal and of hypophysectomized rats were incubated at 37° for various times before centrifuging at 15 000 g to remove nuclei and mitochondria, and Fig. 2 shows the subsequent incorporation of radioactive leucine into the proteins of the cell-free systems prepared from these preincubated homogenates. It is clear that in both instances rapid inactivation has taken place during preincubation. When, however, microsomes from a preincubated homogenate were incubated with soluble fraction prepared from a liver homogenate which had not been preincubated,

Fig. 2. Specific activities of the proteins of the cell-free systems after incubation with DL-[1-¹⁴C]leucine for 30 min. at 37°. The systems were prepared from livers of normal (O) and hypophysectomized (●) rats after preincubation of the whole homogenate at 37° for various times. The conditions of incubation are as in Fig. 1.

almost normal rates of incorporation of amino acid into microsomal protein were observed. The results obtained when microsomes which had not been preincubated were incubated with soluble fraction which had been preincubated confirmed that it was indeed mainly the soluble fraction that was being altered by preincubation in these experiments, and not the microsomes. Table 3 presents these results, and also those of an experiment in which the preincubation was carried out at different stages in the preparation of the microsomes and soluble fraction. Four procedures were used. In procedure 1 the microsomes and soluble fraction were prepared without preincubation; in procedure 2 the whole homogenate was incubated at 37° for 15 min.

before the microsomes and soluble fraction were separated from the rest of the liver brei. In procedure 3 the homogenate was centrifuged at 15 000 *g*, to remove debris, nuclei and mitochondria, the supernatant fluid preincubated for 15 min. at 37° and then the microsomes and the soluble fraction were separated from one another by centrifuging at 105 000 *g*. Finally, in procedure 4 the soluble fraction and a suspension of the microsomes in the buffered medium were prepared and then incubated separately at 37° for 15 min.

Table 3 shows that a large diminution in incorporation of amino acid into protein results from procedure 2. The microsomes were not greatly affected by this preincubation, but the soluble fraction lost much of its ability to prepare amino acids for incorporation. The ability of the microsomes to incorporate amino acids was depressed to a greater extent by procedure 3 than by procedure 2, but the soluble fraction was less affected by procedure 3 than by procedure 2. Procedure 4 caused the greatest depression in the incorporating ability of the microsomes and the least inhibitory effect on the soluble fraction.

The presence of the soluble fraction during preincubation thus appears to exert a protective influence on the microsomes, but the presence of other cell fractions enhanced the inhibition suffered by the soluble fraction during preincubation.

Fig. 3. Specific activities of microsomal protein from livers of normal (O) and hypophysectomized (●) rats after incubation with DL-[1-¹⁴C]leucine at 37° for 30 min. under the standard conditions (see legend to Fig. 1). The microsomes had been preincubated at 37° in the buffered medium for the periods shown.

Sachs (1957) has evidence that the soluble fraction protects the microsomes against freeze-drying as well as against preincubation.

The diminution in incorporating ability suffered by a suspension of the microsomes in buffered medium when preincubated alone at 37° (procedure 4) is depicted in Fig. 3, and it is clear from the results that the effect of preincubation on microsomes from hypophysectomized rats is no greater than the effect on microsomes from normal rats.

Optimum conditions for incorporation. Sachs (1957) has shown that the concentration of ATP, creatine phosphate and GSH in the incubation mixture, and the concentration of protein in the microsomal and soluble fractions, are important factors in determining the amount of amino acid incorporated into protein in his freeze-dried system. Experiments have now been carried out with this system which show that the optimum concentrations of ATP, creatine phosphate and GSH are not greatly different for preparations from normal and from hypophysectomized rats, but that there are different optimum concentrations of the protein fractions.

Fig. 4. Specific activities of microsomal protein from the livers of normal (O, Δ) and hypophysectomized (●, ▲) rats after incubation for 30 min. at 37° with L-[G-¹⁴C]leucine under the standard conditions. In one experiment (Δ, ▲) various amounts of microsomal protein were incubated with a constant amount of soluble-fraction protein and in the other (O, ●) the amount of soluble-fraction protein incubated varied while the microsomal protein content was kept constant. The conditions of incubation are as in Table 1.

Fig. 4 shows the effect of incubating different amounts of microsomal protein with a constant amount of soluble-fraction protein, and different amounts of soluble-fraction protein with a constant amount of microsomal protein. An optimum concentration for both microsomal protein and soluble-fraction protein is found with systems prepared from livers of normal and hypophysectomized rats (cf. Sachs, 1957), but the optima occur at lower protein concentrations with the preparations from hypophysectomized rats. Nevertheless, the differences in incorporation between normal and hypophysectomized rats observed cannot be explained only in terms of differences in the optimum concentrations of proteins, since, as is clear from Fig. 4, the incorporation of amino acids into microsomal protein from hypophysectomized rats was less than that into microsomal protein from normal rats even when the concentration of the former was optimum.

The results of these experiments support the view that the difference in the rate of incorporation of amino acids into protein *in vitro* observed between preparations from normal and hypophysectomized rats is not the result of changes in the microsomes which occur after removal of the liver from the animal, nor is it caused by any difference in the conditions needed by the system from hypophysectomized rats to achieve optimum incorporation of amino acids.

Effect of food intake. Hypophysectomized rats eat less than intact ones, and the amount of food eaten daily can influence the rate of amino acid incorporation into protein and the rate of protein biosynthesis. For example, Rutman, Rutman & Tarver (1955) have shown that changes in the diet influence the rate of incorporation of radioactive methionine into liver slices. It is possible that hypophysectomy is not affecting the micro-

somes directly but that the changes are secondary to changes in food intake.

The experiments described in Table 1 have therefore been repeated with groups of rats all the members of which ate the same amount of the same diet; the results obtained were similar to those obtained with rats which were allowed to eat freely. Differences in food intake between normal and hypophysectomized rats cannot therefore explain the differences in incorporation of amino acid into protein.

Table 4 shows the results of an experiment in which normal and hypophysectomized rats were kept on the same ration for a week and then starved for 18 hr. before preparation of the cell-free system. There is little difference between the starved animals and the others in the amounts of amino acid incorporated into protein. Krah1 (1956) believes that the incorporation of amino acid into rat-liver slices from normal and hypophysectomized rats varies directly with the glycogen content of the slices. The glycogen content of the livers of the animals used in the experiments of Table 4 was determined and, as expected, the starving animals, both normal and hypophysectomized, had almost none. Yet in both groups only small differences in incorporation of amino acid were associated with starvation. The glycogen content of the liver appears not to affect the rate of incorporation of amino acids into protein in the cell-free system.

Treatment of rats with growth hormone. Table 5 shows the results of experiments in which female rats, both normal and hypophysectomized, were treated with growth hormone, and the incorporation of amino acid into their microsomal protein was measured in the cell-free system. It is clear that the effect of hypophysectomy on the microsomes can be suppressed, at least in part, by treatment with growth hormone. The earliest significant effect ($P < 0.01$) of growth hormone so far detected was when 0.5 mg. was given 20 hr. before killing the animal. Intravenous injection of growth hormone 1 hr. before death, and subcutaneous injections 3, 6 and 12 hr. before death, produced no significant change in incorporation of amino acids into proteins in the cell-free system. The effect on normal rats of treatment with growth hormone is also shown in Table 5. It is clear from experiments of the type described above that growth hormone can cause increased incorporating ability in normal microsomes. The relevant results are illustrated in Fig. 5. Treatment of normal and hypophysectomized rats with growth hormone appeared to cause a small increase in the ability of the soluble fraction of the liver to prepare amino acids for incorporation into protein in the microsome, but these changes are not statistically significant. In no experiment did treatment of

Table 4. *Specific activity of microsomal protein from normal and hypophysectomized rats which had been fed, or starved for 18 hr. after incubation under identical conditions with L-[G-¹⁴C]leucine for 30 min.*

The conditions of incubation are as in Table 1. The specific activities are calculated from the formula given under Methods.

	Specific activity of microsomal protein (mean $\pm$ S.E.M.) (counts/min./mg.)
Normal rats	
Fed	133 $\pm$ 8.6
Fasted 18 hr.	121 $\pm$ 6.1
Hypophysectomized rats	
Fed	59 $\pm$ 5.9
Fasted 18 hr.	52 $\pm$ 5.1

Table 5. *Effect of various doses of ox growth hormone in female rats on the specific activity of microsomal protein after subsequent cell-free incorporation experiments with L-[G-¹⁴C]leucine*

The conditions of incubation are as in Table 1. The specific activities are calculated from the formula given under Methods.

	Dose of growth hormone (mg./day)	Time of treatment (days)	Specific activity of microsomal protein (mean $\pm$ S.E.M.) (counts/min./mg.)
Hypophysectomized rats	Nil	—	82 $\pm$ 5.5
	0.5*	1 hr.	77 $\pm$ 6.1
	0.5	1	106 $\pm$ 6.0
	0.02	3	116 $\pm$ 7.1
	0.2	3	152 $\pm$ 10.5
	0.2	7	160 $\pm$ 9.9
Normal rats	Nil	—	189 $\pm$ 11.2
	0.02	3	196 $\pm$ 10.1
	0.2	3	211 $\pm$ 11.1
	0.2	7	239 $\pm$ 8.9

* Intravenous injection.

Fig. 5. Histogram showing the specific activity of microsomal protein after incubation for 30 min. at 37° with DL-[1-¹⁴C]leucine, of microsomes from normal and hypophysectomized rats with their own and one another's soluble fraction. M_N, microsomes from normal rats; M_H, microsomes from hypophysectomized rats; S_N, soluble fraction from normal rats; S_H, soluble fraction from hypophysectomized rats; the additional subscript G signifies that the rat was treated with 0.4 mg. of growth hormone each day for 4 days. Conditions of incubation are as in Table 1. Microsomal specific activity was calculated from the formula given under Methods.

hypophysectomized rats with growth hormone alone succeed in completely reversing the depressing effect which hypophysectomy has on the incorporation of amino acids into microsomal protein *in vitro*.

DISCUSSION

Some steps in the sequence of reactions which results in the incorporation of amino acids into microsomal protein are known. The amino acids are activated by specific amino acid-activating enzymes, which exist in the soluble fraction of the cell and which are precipitated at pH 5.0 (Hoagland *et al.* 1956; Nisemann, Bergmann & Berg, 1957; Lipmann, 1958). Activation requires ATP, and, since pyrophosphate is eliminated in the process, it is suggested that an amino-acyl adenosine monophosphate complex is formed on the surface of the activating enzyme. Hoagland *et al.* (1957) have good evidence that the next step is the formation of a complex between the activated amino acid and the rather low molecular-weight (10–20 $\times 10^3$) soluble RNA of the cytoplasm. Guanosine triphosphate is needed in the next step, in which the amino acid enters the microsome (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958) attached to all or part of the soluble RNA (Crick, 1958). Littlefield, Keller, Gross & Zamecnik (1955) have provided evidence that the assembly of amino acids into polypeptide chains occurs in the microsomal particles (Palade, 1956) of the microsomal fraction. It is postulated (Littlefield *et al.* 1955; Littlefield & Keller, 1957) that the RNA of these particles functions as a template on which the peptide is formed, and schemes can be constructed to account for the way in which the amino acids fall on specific points on the template (Crick, Griffith & Orgel, 1957) so that the finished peptide which peels off the template has a specific amino acid sequence.

Hypophysectomy and amino acid-incorporating ability. It is clear from the experiment described in this paper that hypophysectomy impairs the amino

acid-incorporating ability of the cell-free system and that the main site of the defect is in the microsomes. Any change in the activity of the amino acid-activating enzymes as a result of hypophysectomy is not responsible for the depression in amino acid incorporation found, for the enzymes of the soluble fraction from hypophysectomized rats can work as well as those from normal rat liver if presented with normal microsomes. Similarly a diminution in the ability of the soluble RNA to form a soluble RNA-amino acid intermediate, of the type suggested by Hoagland *et al.* (1957), or a decrease in the ability to transfer the amino acids from the soluble RNA to the microsomal particles, cannot be responsible for the lower rate of incorporation of amino acid found in preparations from the hypophysectomized rats. It is possible that changes have occurred in the activity of these enzymes and carriers, of such a nature that these reactions are not limiting and are not detectable by these experiments. The conditions used in these experiments are such that excess energy is available throughout in a suitable form, so that any effect of hypophysectomy on the formation *in vivo* of ATP for these processes would be masked. Whether or not hypophysectomy brings about changes in cell permeability to metabolites of importance in protein synthesis likewise is not revealed in these experiments. In other words, although the incorporating ability of the microsomes is affected by hypophysectomy in such a way as to reduce the rate at which protein is synthesized, the possibility has not been excluded that other mechanisms, not detected by the system used here, also contribute to the fall in the overall rate of protein synthesis observed directly in the livers of rats deprived of their pituitary gland (Simpson *et al.* 1949).

The results reported here do not support the possibility that the fall in the ability of microsomes to incorporate amino acids into protein which follows hypophysectomy in the rat is caused by a greater degradative change occurring in such microsomes after removal of the liver from the rat. They have also shown that the fall cannot be explained by the alteration in the optimum conditions of the system prepared from the hypophysectomized rat. It is concluded that an effect on the microsomes has occurred *in vivo* as a result of hypophysectomy and that this has reduced the ability of the microsomes to incorporate amino acids into protein.

Effect of amount of food eaten on protein biosynthesis. Hypophysectomized rats eat less than normal ones but this difference in food intake cannot, by itself, account for the diminution in incorporating ability of the microsomes from livers of hypophysectomized rats, for similar differences in incorporating ability between microsomes from

livers of normal and hypophysectomized rats were found whether the rats had been fed on the same ration or had been allowed to eat freely. Moreover, starvation for 18 hr. did not materially alter the ability of the microsomes from livers of normal or hypophysectomized rats to incorporate amino acid into protein. Clark, Naismith & Munro (1957) have shown that starvation for 18 hr. of rats, previously fed on a diet containing protein, causes a fall in the RNA content of the liver. The experiment described in Table 4 shows that the incorporating ability of the microsomes is not altered by this treatment. It can be concluded, either that the RNA lost by 18 hr. starvation is not concerned in protein biosynthesis, or that microsomal protein is lost during starvation for 18 hr. at such a rate that the ratio of microsomal protein to reactive microsomal RNA is kept constant. If this occurs, when similar amounts of microsomal protein from fed and starving rats are used in incubation experiments, they will contain similar amounts of reactive microsomal RNA and, therefore, similar amounts of amino acid will be incorporated into protein. The number of microsomes per liver may, however, diminish as a result of starvation.

Effect of liver-glycogen content on protein biosynthesis. Krah1 (1956) found that the amount of amino acid incorporated into the protein of rat-liver slices from hypophysectomized rats was proportional to the glycogen content of the slices. Incorporation of amino acid in the cell-free system in the experiments described in this paper is not related directly to the liver-glycogen content, for similar incorporation was found in the microsomes from fed and from starved rats. These results are not necessarily at variance with one another; for, if the glycogen content of the liver is an index of the amount of glucose available for RNA template formation, or for energy production, then it could be argued that in the experiments with cell-free preparations the amount of amino acid incorporated will depend on the preformed template, but that in slices more template could be formed, or more energy made available, during the experimental period.

Effect of growth hormone on protein biosynthesis. It is encouraging that the results of the experiments *in vitro* reported here agree with the expectations one derives from effects which hypophysectomy and treatment with growth hormone are known to have on protein synthesis *in vivo*. Hypophysectomy of the rat is followed by a loss of body weight and of the protein of the carcass (Lee & Ayres, 1936) and treatment with crude (Lee & Schaffer, 1934) or purified (Simpson *et al.* 1949) growth hormone can reverse or prevent this process. Growth hormone can cause retention of nitrogen in many normal animals (see Ketterer *et al.* 1957), and can stimulate

the livers of hypophysectomized rats to synthesize more serum albumin (Ulrich, Tarver & Li, 1954). Russell's (1951, 1955) evidence indicates that growth hormone acts by stimulating protein synthesis rather than by inhibiting protein breakdown, and a similar conclusion was reached by Friedberg & Greenberg (1947) as a result of experiments on the incorporation of radioactive amino acids into protein in the muscles of mice.

In the experiments described here, it is shown that growth hormone is one of the factors determining the ability of the microsomes of rat liver to incorporate amino acids. Complete quantitative reversal of the effects of hypophysectomy was not obtained with growth hormone alone, probably because other hormones of the pituitary gland play some part, directly or indirectly, in the reactions which bring about protein biosynthesis. The nature of the changes in the microsomes which occur as a result of hypophysectomy are being studied.

SUMMARY

1. Less radioactive amino acid was incorporated into protein *in vitro* in a cell-free system (Zamecnik & Keller, 1954) prepared from the livers of hypophysectomized rats than was incorporated into protein in a similar system prepared from the livers of normal rats.

2. It was shown that changes in the activity of the amino acid-activating enzymes of the soluble fraction of the cell or in the ability of the soluble ribonucleic acid to form amino acid-soluble ribonucleic acid intermediates were not responsible for the diminished incorporation of amino acid into protein found in this system as a result of hypophysectomy. The microsomes from the livers of hypophysectomized rats were, however, less able than those from normal rats' liver to incorporate the activated amino acids into protein.

3. The diminished ability of the microsomes to incorporate amino acids into microsomal protein shown by liver preparation from hypophysectomized rats could not be attributed to (a) greater breakdown of the microsomes during incubation by more extensive ribonuclease or proteolytic-enzyme activity, (b) differences in the optimum conditions needed by the preparations from differently treated rats, or (c) the smaller food intake of hypophysectomized rats compared with that of normal rats.

4. Starvation of normal or of hypophysectomized rats for 18 hr. did not alter the ability of the liver microsomes to incorporate amino acids into protein. Incorporation of amino acid into protein in this system is not related to the glycogen content of the liver.

5. Treatment of the hypophysectomized rats with ox growth hormone could restore, in part, the

ability of their liver microsomes to incorporate amino acids into protein. Other pituitary hormones may also be concerned in these processes. Treatment of normal rats with growth hormone increased the ability of the liver microsomes to incorporate amino acid into protein.

6. It is concluded that hypophysectomy of the rat results in changes in the liver microsomes such that they are less able to incorporate amino acids into protein than liver microsomes from normal rats and that treatment with growth hormone can partly reverse such changes. In this way the depressed rate of protein biosynthesis observed *in vivo* in the livers of hypophysectomized rats may be at least partly accounted for.

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Incorporation of Sodium [1-¹⁴C]Hexanoate and Sodium Hydrogen [¹⁴C]Carbonate into Milk Constituents by the Perfused Cow's Udder

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The occurrence of a C₆ volatile acid was observed in the ox rumen by McClymont (1951) and in the sheep rumen by Gray, Pilgrim, Rodda & Weller (1952). The metabolism of [1-¹⁴C]hexanoate and [2-¹⁴C]hexanoate in the intact dairy cow was studied by Kleiber and his collaborators. Most of the ¹⁴C activity, recovered in milk constituents, was present in the lactose, smaller quantities of ¹⁴C being found in the fat and casein. The specific activity of plasma glucose, after injection of [1-¹⁴C]hexanoate, was much higher than that of acetate. It was concluded from this observation that the transfer of carbon derived from hexanoate to glucose mostly bypasses the plasma acetate pool, even though acetate can contribute carbon to plasma glucose. A similar conclusion was reached from the labelling of plasma acetate and glucose after injection of ¹⁴C-labelled butyrate (Kleiber, 1958).

In the experiments described here we have studied the incorporation of sodium [1-¹⁴C]hexanoate into different constituents of milk and tissue by the perfused cow's udder. The results indicate that the udder has the capacity to degrade hexanoate to acetyl-coenzyme A. This hexanoate does not behave in a glycogenic manner.

In a previous experiment it was shown that ¹⁴CO₂ was incorporated only to a negligible extent into fatty acids and lactose by the perfused-udder preparation (Cowie *et al.* 1951). No information is available about the incorporation of carbon dioxide into the amino acids of casein by the same preparation. For these reasons a perfusion experiment was performed in the presence of sodium hydrogen [¹⁴C]carbonate and the incorporation into amino acids was studied.

EXPERIMENTAL

Perfusion of half-udders. Each experiment was carried out on one lactating half-udder perfused during 150 min. by the method of Peeters & Massart (1952). Half-udders from different cows were utilized. Experimental details of the perfusion technique and of ¹⁴CO₂ collection were as described by Verbeke, Lauryssens, Peeters & James (1959). Immediately before slaughter the cows were milked out as completely as possible with the aid of intravenously injected oxytocin. The udders were removed as soon as the cows were shot. The glands were completely bisected along the median septum and one half was connected to the perfusion apparatus. Heparinized, fresh cow's blood (9 l.) was used for the perfusion of one half.

Sodium [1-¹⁴C]hexanoate (0.2 m-mole = 1 mc) was dissolved in 50 ml. of water and added to the blood 30 min. after the beginning of perfusion. The experimental animal had yielded approx. 18 l. of milk daily. After the administration of labelled substrate, inactive acetate (sodium acetate trihydrate, 5.25 g. in 250 ml. of water) was introduced regularly into the blood reservoir by means of a constant-drip device. At the end of the experiment oxytocin (10 i.u.) was injected into the udder artery to cause ejection of milk: 290 ml. of milk was collected. Skin, teats and adipose tissue were removed carefully by dissection. The udder tissue was weighed (wt. 3.88 kg.), cut into pieces and cooled at -15° for 1 hr.

Sodium hydrogen [¹⁴C]carbonate (5.9 mg. = 1 mc) was added to a solution of inactive sodium acetate trihydrate (10.5 g. in 750 ml. of water). This solution was added to the blood reservoir by means of a constant-drip device started 30 min. after the commencement of perfusion. The experimental animal had previously yielded approx. 12 l. of milk daily. After perfusion 150 ml. of milk was collected.

Isolation of udder tissue and milk-fat fractions. Udder tissue and milk-fat fractions were isolated after perfusion with [1-¹⁴C]hexanoate as described previously (Verbeke *et al.* 1959).