Hobson & Rees, 1955) and served to identify them by synthetic as opposed to degradative means. Throughout the species investigated the phosphokinases predicted by the previous results were all proved to be present and capable of phosphorylating the expected base.

As was reported for some of the echinoderms (Needham & Baldwin, 1937), several annelids, e.g. *Glycera gigantea*, *Nereis diversicolor* and *Myxicola infundibulum*, contain a mixture of phosphagens and show that the enzyme systems for the synthesis and utilization of phosphagens in living muscle are completely duplicated.

The experimental results again emphasize that creatine phosphate, acting as a phosphagen, is not confined to the echinoderms, protochordates and vertebrates, but is also present, often in considerable quantities, in the annelids. At the present time the distribution of creatine phosphate appears quite arbitrary. This fact lessens the value of the identification of the phosphagens in evolutionary studies in the animal kingdom.

SUMMARY

1. A method of extraction of guanidine phosphokinases from annelid muscle is described.

2. Transphosphorylation has been demonstrated and the synthesized guanidine phosphates have been identified.

3. It is concluded that taurocyamine and glyco-cyamine phosphate as well as creatine phosphate can act as phosphagens in certain of the annelids.

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Protein Synthesis in Guinea-pig Liver

INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO PROTEINS OF THE MICROSOME FRACTION *IN VIVO*

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When a labelled amino acid is injected intravenously into normal animals the liver protein becomes rapidly labelled. Fractionation of the liver subcellular components by differential centrifuging has shown that there is a high rate of incorporation of amino acid into the proteins of the microsome fraction (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950; Keller, 1951). It has been shown, also, that most of the cytoplasmic ribonucleic acid is concentrated in the microsome fraction and that, at least with pancreas, digestion of the microsomes by ribonuclease releases a protein with a particularly high turnover rate (Allfrey, Daly & Mirsky, 1953).

The present investigation was designed to gain additional information concerning the role of the microsome fraction in protein synthesis. It was found, following a suggestion by Dallam (1955), that further fractionation of the microsome material of guinea-pig liver could be achieved by extraction with solutions of varying ionic strength and pH. This method was used to follow the rate of incorporation of radioactive amino acids into different microsome proteins after injection of a mixture of [¹⁴C]amino acids into normal guinea pigs. Our results indicate that the microsome fraction contains a complex mixture of proteins with widely differing turnover rates, the protein fraction with the highest turnover

rate being associated with ribonucleic acid. This connexion between rapid protein turnover and attachment to ribonucleic acid may be significant, since two other laboratories have obtained rather similar results using different methods of fractionation (Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955). A preliminary account of the present investigation has been published (Simkin, 1955).

EXPERIMENTAL

Chemical analyses

Total nitrogen. Estimations were by a micro-Kjeldahl procedure.

Protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), whole microsome protein being used as standard (the proteins of all fractions gave the same optical density value per unit weight with the reagent).

Ribonucleic acid. The orcinol method of Meibbaum (1939) was used to estimate ribonucleic acid (RNA). A sample of guinea-pig liver cytoplasmic RNA, isolated by the method of Davidson, Frazer & Hutchison (1951), was used as standard.

Deoxyribonucleic acid. The diphenylamine reaction as described by Burton (1955) was used for estimation of deoxyribonucleic acid (DNA).

Phosphorus. The method of Allen (1940) was used, but with the total volume scaled down to 5 ml.

MATERIALS

Animals. Guinea pigs from the Institute stock were employed (750–900 g., about 7 months old). The animals were starved overnight before the experiment. All injections were intravenous.

Radioactive amino acids. A mixture of uniformly labelled L-[¹⁴C]amino acids was prepared by acid hydrolysis of [¹⁴C]chlorella protein; the latter was supplied by the Radiochemical Centre, Amersham, Bucks. Each animal received about 35 μ C/kg. (7.5 μ moles of N/kg.).

Preparation of homogenates

Removal of liver. For the 2 min. experiment, the method was as follows: the dose was injected over a period of 5 sec. and the animal killed by a blow on the head 120 sec. later. The body was plunged into a bath of ice-cold physiological saline, the abdominal wall was opened immediately and the liver removed. The whole operation required 150 sec. from the completion of the injection. For experiments at longer time intervals, the cold bath was not used but the liver was quickly transferred to ice-cold saline. All homogenates and subcellular fractions were maintained at 0–5° throughout.

Method of homogenizing. The liver was minced and freed from connective tissue by forcing through a metal screen with holes of 1 mm. diameter. The liver mince (approximately 25 g.) was mixed with an equal weight of 0.25 M sucrose, and homogenized in a Potter-Elvehjem type homogenizer, with a polytetrafluoroethylene pestle (referred to below as a Teflon homogenizer). The difference between

the internal diameter of the tube and the diameter of the pestle was 0.25 mm. The pestle was driven at 2000 rev./min. and the liver was homogenized for 45 sec.; about 15–20 up-and-down strokes of the pestle were completed during this period. Twice the original volume of 0.25 M sucrose was then added to the homogenate and a sample (2 ml.) was taken for determination of the radioactivity of the whole liver protein.

Preparation of the microsome fraction and the cell sap

Whole cells, nuclei and mitochondria were removed from the diluted sucrose homogenate by centrifuging in a refrigerated, angle-head centrifuge (centrifuge B). The homogenate was spun for 10 min. at 7000 rev./min. This corresponded to 5000 g ($g_{\max}$) at the maximum radius ($r=9.5$ cm.) and 4000 g (g_{av}) at the average radius. The supernatant was removed by suction, the small amount of supernatant immediately above the sediment being rejected. The supernatant was then recentrifuged for 15 min. at 8200 rev./min. ($g_{\max}=7000$; $g_{\text{av}}=5500$). The supernatant was collected, a small amount of supernatant overlying the sediment being discarded. At this stage only a few particles of the size of mitochondria could be found in the supernatant (ultraviolet phase-contrast microscopy). The supernatant was diluted to 100 ml. with 0.25 M sucrose (this involved diluting with slightly less than an equal volume of sucrose). The diluted supernatant was then centrifuged in a Spinco Model E ultracentrifuge, with preparative rotor A, for 45 min. at 42040 rev./min. ($g_{\text{av}}=103000$). The supernatant was removed by suction, that part of the supernatant immediately above the sediment of microsome material being rejected. The supernatant obtained at this stage is referred to hereafter as the cell-sap fraction.

Preparation of washed microsomes. The microsome pellet separated from the cell sap was washed twice with 2–3 ml. of 0.25 M sucrose, by layering sucrose over the pellet, inverting the tube and allowing to drain. The microsome fraction was then washed twice with glycerol, as suggested by Dallam (1955). Aqueous glycerol was used at this stage to obviate the need for ultracentrifuging. Often a trace of brown-coloured material was seen underlying the pink microsome material (this brown material is presumably of mitochondrial origin; cf. Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). It was possible to reject this material while removing the microsome sediment completely from the centrifuge tube. The microsome fraction was dispersed in about 10 ml. of 38% (v/v) glycerol (A.R.) by a Teflon homogenizer driven at 1000–1500 rev./min. The suspension was diluted to 50 ml. by the addition of 38% glycerol, and then centrifuged in centrifuge B for 35–40 min. at 17000 rev./min. ($g_{\max}=30000$, $g_{\text{av}}=25000$; deceleration from such speeds took about 10 min.). Sometimes a small amount of material (estimated at about 5% or less) failed to sediment. The supernatant, containing the unsedimented material, was discarded. The sediment was washed again in a similar manner. Complete sedimentation was always achieved in the second washing. The supernatant was discarded and the centrifuge tubes containing the sediment were drained. This sediment will be referred to as the washed microsome fraction. A sample of this material was taken for determination of the radioactivity (or analysis) of the whole microsome fraction.

Fractionation of washed microsomes

Immediately before the extraction procedure was begun, glycerol was removed from the surface of the washed microsome sediment by adding about 5 ml. of 0.14 M-NaCl to the centrifuge tube, and pouring off the solution, the whole operation being carried out as rapidly as possible.

Each extraction was carried out by suspending the material in the appropriate solution, a Teflon homogenizer driven at about 1500 rev./min. being used. The suspension was allowed to stand for 15–20 min. and the insoluble material was then removed by centrifuging for 15 min. in centrifuge *B* at 17 000 rev./min. The supernatant was collected by suction.

The details of the fractionation scheme are given in Fig. 1. Usually, fractionation was started with washed microsome material containing about 25 mg. of N. This quantity was obtained from about 15 g. of liver.

In some early experiments, fraction *C* was extracted by suspending insoluble material from the previous step in 0.14 M-NaCl (0.5 ml./mg. of N) and adding an equal volume of 0.01 N-NaOH; the extract had the usual composition of fraction *C*.

Isolation of protein for radioactivity measurement

Protein was precipitated from all samples by the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA); the complete homogenate and whole microsome fraction samples were diluted about 5 times before precipitation. The precipitate was centrifuged down, the supernatant discarded, and the precipitate redissolved in warm N-NaOH (3–10 ml. according to the amount of precipitate). A large excess of an unlabelled amino acid mixture was then added in the form of a protein hydrolysate, 2–5 μ moles of hydrolysate N (0.5–1.0 ml.) being added/ml. of NaOH. The protein was then reprecipitated with TCA. The protein precipitate was washed as follows: once with 5% (w/v) TCA at 20°, once with 5% TCA, heating for 15 min. at 90° before centrifuging, once with 5% TCA at 20°, twice with acetone at 20°, twice with 3:1 (v/v) ethanol-ether at 60° and finally twice with ether at 20°. The protein precipitates were then air-dried, usually at 37°.

As the liver from the animal killed at 2 min. was likely to contain much labelled free amino acids, a TCA solution which contained casein hydrolysate (4 μ moles of N/ml.) was used to precipitate protein in the first step. The remainder of the isolation procedure was as described above.

Fig. 1. Scheme for fractionation of microsome material. The volumes of extracting solutions quoted are given in terms of ml./mg. of nitrogen of the original washed microsome material taken for extraction.

The protein was not isolated from subfractions A_2 and B_2 owing to the low concentration of material contained in these fractions.

Determinations of radioactivity. The dried protein samples were counted on 0.3 cm.² or 1.0 cm.² polythene planchets at infinite thickness, with an end-window Geiger counter.

Analyses of microsomes and microsome subfractions

The method used for isolation of protein for radioactivity determination was designed to ensure absence of contamination by traces of free radioactive amino acids. For analyses of the various microsome subfractions a somewhat simpler procedure was used.

Protein and lipid-soluble phosphorus. A sample of 1.0–1.5 ml. of the appropriate fraction (2.0–3.0 ml. for fractions A_2 and B_2) or of a 1:5 dilution of the whole microsome fraction suspension was taken, and an equal volume of 10% (w/v) TCA added. The precipitates were washed by the use of a procedure similar to that described above for isolation of protein, except that the stage of re-solution in NaOH and reprecipitation was omitted, and the precipitates were washed twice with cold TCA after extraction with hot TCA; 1.5 ml. of extractant was used at each stage. The acetone, ethanol-ether and ether extracts were pooled, the solvents removed *in vacuo*, and the total phosphorus content of the residues was determined (lipid-soluble P). The precipitate remaining after ether extraction was dissolved in warm π -NaOH, and the protein in solution determined.

Estimation of ribonucleic acid and acid-soluble nucleotide. RNA was separated from other constituents in the following manner. An equal volume of cold π -HClO₄ was added to each sample at 0° (volumes as for procedure described immediately above). The precipitate was washed twice with 1.0 ml. of chilled 0.5 π -HClO₄. The original supernatant and the two washings were pooled, and will be referred to as the acid-soluble nucleotide fraction. Lipid was extracted from the precipitate by three washings with 1.0–1.5 ml. of ethanol-ether-CHCl₃ (2:2:1, v/v); the first two extractions were carried out at 20° and the last at 50°. In the first extraction, the solvent mixture was made 0.1 π with respect to potassium acetate. The precipitate was then suspended in 2.0 ml. of π -HClO₄ and allowed to stand at room temperature overnight (cf. Ogur & Rosen, 1950). The precipitate was centrifuged down and washed twice with 1.0 ml. of π -HClO₄. The digest supernatant and the two HClO₄ washings were combined and taken as the RNA digest. The pentose content of both the acid-soluble nucleotide fraction and the RNA digest was determined.

Deoxyribonucleic acid. The separation procedure of Schneider (1945) was applied to the various microsome subfractions and to the washed microsome fraction. For each estimation, the amount of material used was three times that used for RNA estimation. The material extracted by 5% TCA at 90° was assayed for DNA.

Physical methods

Ultraviolet-absorption curves. The ultraviolet-absorption curves of the fractions were determined with a Unicam SP. 500 spectrophotometer.

Electrophoresis and sedimentation studies. Preliminary investigations into the electrophoretic and sedimentation characteristics of fractions A_1 , B_1 and C were carried out. Because of the low concentration of material present in these

fractions, it was necessary to concentrate each fraction several-fold. The fractions were dialysed against appropriate strength veronal-NaCl buffers and the dialysed materials freeze-dried. The concentrated preparations of fractions A_1 and C were examined in a Perkin-Elmer Tiselius electrophoresis apparatus, with veronal buffer of pH 8.4, $I = 0.1$, and a current of 12 ma. The concentrated preparations of fractions A_1 , B_1 and C were analysed in the Spinco Model E ultracentrifuge at 59780 rev./min. with analytical rotor A and a 12 mm. cell, with veronal buffers of pH 8.4; the ionic strengths were 0.15 for fractions A_1 and C , and 0.3 for fraction B .

RESULTS

Incorporation of [¹⁴C]amino acids into the proteins of liver-cell fractions

Table 1 records the radioactivity of proteins from whole liver, washed microsome fraction and cell-sap fraction obtained from guinea pigs killed at different times after the intravenous injection of a mixture of [¹⁴C]amino acids ([¹⁴C]chlorella-protein hydrolysate). During the first 30 min. there is a rapid increase in the radioactivity of the protein of whole liver; thereafter, the increase in activity is more gradual. There is, however, a very rapid initial uptake into the protein of the microsome fraction, which also lasts for about 30 min., this being followed by a period in which there is only a very slow increase in activity; thus, after 2 min., the microsome protein has about four times the activity of the whole liver protein. The soluble protein of the cell-sap fraction has initially a much lower activity than either the whole liver or microsome protein, but the activity gradually increases with time,

Table 1. *Radioactivities of proteins isolated from the livers of guinea pigs killed at various time intervals after the intravenous injection of a mixture of [¹⁴C]amino acids*

L represents the protein of the complete liver; S , that of the cell-sap fraction; M , that of the washed microsome fraction and A , B , C , D and E the proteins of the various microsome subfractions obtained by salt extraction. Under the conditions used, 10^{-3} μ c/mg. of carbon gives 1000 counts/min.

Time after injection (min.)	Radioactivity of protein (counts/min./cm. ²)							
	L	S	M	A	B	C	D	E
2	15	6	56	15	44	48	58	63
5	45	23	128	67	60	182	133	124
15	85	46	220	177	108	445	183	185
30*	136	76	323	348	162	762	285	227
60	167	113	337	309	197	673	349	302
195	184	164	397	248	222	392	408	368

* Each value given, except that for S , is the mean of two values obtained in two separate experiments. The results obtained were in good agreement.

continuing to an appreciable extent after incorporation into the microsome protein has essentially ended. These results are in agreement with the results of Keller, Zamecnik & Loftfield (1954).

Incorporation of [14 C]amino acids into the proteins of microsome subfractions and characterization of subfractions by chemical analysis

Table 1 records also our results on incorporation of [14 C]amino acids into the proteins of the various microsome subfractions obtained by the application of the extraction procedure outlined in Fig. 1 and in the Experimental section. In Fig. 2, the values have been plotted so as to show the specific radioactivity of the protein of the various fractions relative to the activity of the whole microsome protein at corresponding time intervals. It has been found that, whereas changes in the specific activity of the amino acid mixture administered result in different absolute values for the radioactivities of different cell proteins, the ratios of the activities of the various proteins are essentially constant. The method of expression adopted in Fig. 2 will, therefore, largely eliminate variations in activity due to differences in dose. The values given in Table 2 for chemical analyses of the microsome subfractions are those obtained for a single liver preparation; other analyses have shown that the values given may, in general, be taken as typical.

Fraction A. The protein extracted by 0.14M sodium chloride had, shortly after injection of amino acids, a radioactivity considerably lower than the whole microsome protein, but activity increased with time and at 30 min. this protein fraction was slightly more active than the whole microsome protein. Later, activity fell and at 195 min. was 60 % of the activity of the whole microsome protein.

This fraction contained about 10 % of the total nitrogen and 12 % of the protein of the washed-

microsome fraction. No RNA or lipid-soluble phosphorus could be detected. Most of the material extracted by 0.14M sodium chloride was present in the first of the two extracts (Fig. 1). The second extract contained only about one-third of the material present in the first.

Fraction B. The protein of the fraction extracted by M sodium chloride had (except at 2 min.) an activity approximately one-half of that of the whole microsome protein.

Fig. 2. Change of radioactivity of proteins of microsome subfractions with time after injection of radioactive amino acids. The radioactivity is expressed as the ratio of the specific activity of the subfraction protein (X) to that of the whole microsome protein (M). The curve for the protein of fraction E has not been given, as it was similar to that found for fraction D .

Table 2. *Analysis of fractions obtained from guinea-pig liver microsome material*

The fractions were prepared from a washed microsome fraction as described under Methods, except that a smaller quantity of NaOH was used to effect the separation of fractions D and E (see text).

Fraction	Percentage of complete microsome fraction				$\frac{\text{RNA}^\dagger}{\text{Protein}}$
	Total N	Protein	RNA*	Lipid-soluble P†	
A_1	7.6	9.9	—	—	—
A_2	2.4	2.4	—	—	—
B_1	9.9	10.1	18.2	Trace	0.21
B_2	3.4	3.2	6.7	Trace	0.24
C	11.8	12.3	43.2	Trace	0.41
D	11.4	14.1	21.4	8	0.17
E	53.6	48.0	10.4	89	0.024

* RNA values have been corrected for degradation occurring in cold perchloric acid (see text).

† Traces of lipid-soluble P appeared to be present in fractions B_1 , B_2 and C , but the amounts were too small to be determined with precision.

‡ The RNA/protein ratio of the complete microsome material was 0.114.

This fraction contained about 13 % of the total nitrogen, 13 % of the protein, and in the experiment detailed in Table 2, 25 % of the RNA of the whole microsome fraction. The RNA/protein ratios were 0.21 for the first extract and 0.24 for the second. In other experiments, values of 0.60 and 0.45 have been obtained for the RNA/protein ratio of fraction *B* and the proportion of the total RNA in *B* was rather higher, the RNA value for fraction *C* then being correspondingly lower. The second M sodium chloride extract contained about one-third of the material found in the first. No more than trace quantities of lipid-soluble phosphorus were detected.

Fraction C. The protein of this fraction, extracted by buffer, pH 9, increased rapidly in radioactivity to a maximum value, more than twice that of the whole microsome protein, at about 30 min. The activity then fell rapidly and at 195 min. this fraction was no more active than the whole microsome protein.

Fraction *C* contained about 12 % of the total nitrogen, 12 % of the protein and 43 % of the RNA of the complete microsome fraction. The RNA/protein ratio was 0.41. In other experiments, ratios of 0.27 and 0.32 have been recorded. Lipid-soluble phosphorus was present only in trace quantities.

Fractions D and E. The proteins of these fractions (separated by solubility in 0.01 M sodium hydroxide) showed a similar pattern of labelling to one another. The initial rate of increase in activity was less than that for whole microsome protein, but after 30 min., as the activity of fraction *C* declined, these two fractions continued to increase in activity, and eventually they reached an activity approximately equal to the whole microsome protein.

Fractions *D* and *E* represent together about 65 % of the total nitrogen, 62 % of the protein, 32 % of the RNA and essentially all of the lipid-soluble phosphorus of the microsome fraction. The division between fractions *D* and *E* is somewhat arbitrary, depending upon the quantity of alkali added per unit weight of material. In the experiment recorded in Table 2, 0.6 ml. of 0.01 N sodium hydroxide/mg. of nitrogen was used; in other experiments 1.0 ml. was used, and a considerably greater proportion of lipid-soluble phosphorus and protein was brought into solution, approximately equal quantities of protein and lipid-soluble phosphorus being found in fractions *D* and *E*. With 0.6 ml. of 0.01 N sodium hydroxide/mg. of nitrogen, the RNA/protein ratio of fraction *D* was 0.17; with larger quantities of sodium hydroxide, the ratio was lowered. With progressive rise in the pH of the extractant, the remaining RNA is readily dissolved, together with increasing amounts of protein and lipid-soluble phosphorus, until only a small insoluble residue of protein remains (cf. Dallam, 1955).

Deoxyribonucleic acid

Neither the whole microsome fraction nor any of the fractions *B*, *C*, *D* or *E* when tested separately contained a quantity of DNA detectable by the method of analysis employed. Fraction *A* was not tested.

Characterization of microsome subfractions by physical methods

Ultraviolet-absorption curves. The ultraviolet-absorption spectra of the fractions (Fig. 3) confirmed the results of chemical analyses. The curve obtained for fraction *A* resembled that given by a protein solution, whereas curves found for fractions *B* and *C* indicated the presence of relatively large quantities of nucleic acid. Because of the characteristic differences found, the absorption spectra of the fractions were always determined, providing a useful means of following the progress of the fractionation procedure and checking on the nature of the extracts obtained.

Electrophoresis and sedimentation. Fractions *A* and *C* when analysed by electrophoresis at pH 8.4 as described in the Experimental section showed complex, though dissimilar, patterns, indicating the presence of numerous components. In the analytical ultracentrifuge, at pH 8.4, both *A* and *C* showed a rapidly moving peak ($S_{20} = 7-8$) and also a slower-moving more diffuse peak ($S_{20} = 3-4$). When fraction *B* was dialysed and freeze-dried, a substantial part of the material failed to redissolve in buffer (with fractions *A* and *C* almost all the freeze-dried material was soluble). As a consequence, it was difficult to obtain a solution of sufficiently

Fig. 3. Ultraviolet-absorption curves for microsome sub-fractions in aqueous solution.

high concentration for electrophoresis or ultracentrifuging. The pattern obtained in the ultracentrifuge at pH 8.4 suggested that the soluble material contained a large number of components of comparatively low molecular weight. No peak was found corresponding to the more rapidly moving component of the other two fractions.

DISCUSSION

Analytical methods. Standard methods of chemical analysis were quite satisfactory when applied to the whole microsome fraction, but difficulty was experienced when these methods were applied to the isolated microsome subfractions. Determination of RNA, by either ultraviolet absorption at 260 m μ . or estimation of phosphorus or pentose, showed that the RNA of the microsome subfractions was particularly labile and that upon contact with perchloric acid at 0° for 60 min. or less, some of the RNA was degraded into soluble fragments. In the experiment described in Table 2, this amounted to 26 % of the RNA. A small quantity of nucleotide material was extracted from the whole microsome fraction by cold perchloric acid (cf. Siekevitz, 1955), but this was much less than was obtained by similar extraction of a corresponding quantity of the microsome subfractions. In consequence, RNA values for fractions *B*, *C*, *D* and *E* have been calculated as the sum of the acid-soluble nucleotide plus the usual perchloric acid digest (RNA digest). This procedure appears to be justified, for in the values given in Table 2 the sum of the acid-soluble nucleotide plus the RNA digest nucleotide for fractions *B*, *C*, *D* and *E* was 97 % of the nucleotide content of the RNA digest of the whole microsome fraction.

Difficulty was also encountered in the isolation of protein from fraction *A*. Thus it was found that the protein of fraction *A* was largely soluble in 80 % (v/v) ethanol (method of Schneider, 1945) and was not reprecipitated completely on acidification after digestion with sodium hydroxide (method of Schmidt & Thannhauser, 1945).

Fractionation procedure. At first sight, the fractionation scheme (Fig. 1) used in this investigation may appear to be somewhat arbitrary, but preliminary experiments showed that, in fact, the conditions were rather critical and that the fractionation could be reproduced readily from one experiment to another. The success of the method depends upon the solubility characteristics of the various subfractions, and these differ rather sharply; thus a single extraction with 0.14M sodium chloride extracted most of fraction *A* and a second extraction with the same solvent dissolved relatively little more protein. The distinction between fractions *B* and *C* depended almost entirely on control of pH, *B*

being soluble in M sodium chloride at pH 7 (0.01M phosphate buffer being used to control the pH). Further extraction at this pH or even at pH 8.3 (0.05M bicarbonate buffer) with M sodium chloride dissolved little further material, but at pH 9 it was possible to extract fraction *C* with 0.1M bicarbonate-carbonate buffer. As already indicated in the Results section, the separation between fractions *D* and *E* is much less well defined and they can hardly be regarded as distinct entities.

The present procedure is a development of a method first suggested by Dallam (1955), but the results obtained in the present investigation differ quantitatively from those which he reported. This difference is probably due to the different nature of our own and Dallam's subcellular fractions. Our guinea-pig liver microsome fraction was isolated by ultracentrifuging using 0.25M sucrose, whereas the 'small-granule' fraction of Dallam was isolated with aqueous glycerol. The 'small-granule' fraction was isolated from 38 % (v/v) glycerol by centrifuging at 12000 *g* for 15 min. We have found that centrifuging for at least 40 min. at 30000 *g* was required for the complete sedimentation from 38 % (v/v) glycerol of the microsome fraction previously isolated from liver at 103000 *g* in 0.25M sucrose. It is thus likely that a large part of the microsome fraction would not sediment from 38 % (v/v) glycerol at the centrifugal force employed by Dallam. The 'large-granule' fraction of Dallam was sedimented from 63 % (v/v) glycerol by centrifuging at 10000 *g* for 10 min. and it is likely that complete sedimentation of the mitochondrial fraction was not achieved; thus Dallam's 'small-granule' fraction would probably contain a considerable quantity of the mitochondrial fraction, and it is not surprising that in further fractionation of this material his results should differ substantially from those reported here.

Distribution of radioactivity between microsome proteins

The results of the present investigation confirm that in the normal animal the liver microsome protein has a much higher turnover rate than the protein of other liver fractions. Perhaps the most striking feature of the present data is the different behaviour of the nucleoprotein fractions *B* and *C*. Fraction *B* protein has a relatively low turnover rate, whereas the protein of fraction *C* reaches maximum activity 30 min. after injection of isotope and is at this time nearly five times as active as the protein of fraction *B*.

While this work was in progress, several quite different methods for the fractionation of microsome material were reported, and it is particularly instructive to compare the results of experiments on amino acid uptake made with these different

methods. Mirsky and his colleagues (Allfrey, Daly & Mirsky, 1953, 1955; Daly, Allfrey & Mirsky, 1955) have found that the protein released together with nucleotides when the microsome fraction of mouse pancreas is treated with ribonuclease has a higher turnover rate than the whole microsome protein; the time of maximum labelling of their fraction is about 1.5 hr. after administration of labelled amino acid. Hultin (1955) used prolonged extraction with 0.1M bicarbonate at pH 8.4 to obtain a liver-microsome subfraction containing nearly all the RNA of the microsomes. The protein of this fraction was more rapidly labelled than that of any other fraction examined. Littlefield *et al.* (1955) treated rat-liver microsomes with deoxycholate, which dissolved much of the lipoprotein present, and they obtained a suspension of ribonucleoprotein particles with a rather similar RNA/protein ratio to that of our fraction *C*. The protein of the ribonucleoprotein granules (which accounted for most of the RNA of the microsomes) had a much higher turnover rate than the protein of the whole microsome fraction and reached its maximum activity only 3 min. after injection of radioactive amino acid.

It is thus clear that protein which is closely associated with RNA can be shown by four different methods to have a higher turnover rate than the protein of the whole microsome fraction. Each method provided a protein fraction with a different turnover rate, but each group of workers has used a different animal species and some of the differences recorded might be due to this. The time of maximum labelling varies over such a large range (3–90 min.), however, that another explanation seems more likely. In the present work, the protein associated with RNA was found to be heterogeneous; the apparent turnover rate of a mixture of different proteins will obviously vary widely according to the proportions in which those proteins are present and would no doubt change greatly with the use of different methods for extraction of ribonucleoprotein. This may well explain some of the divergent results.

Since the proteins of fractions *A* and *C* seem to have a higher turnover rate than the other liver proteins, it is tempting to suggest that they are precursors of other proteins; however, the demonstration that fractions *A* and *C* contain numerous components makes it impossible at present to provide much support for such a postulate, since the turnover rates of the individual components are unknown. It would obviously be desirable in work of this nature to study individual proteins, and some progress along these lines has been made by Daly *et al.* (1955). They found that trypsinogen and chymotrypsinogen of mouse pancreas showed a higher rate of incorporation of amino acid than did a protein fraction released from microsomes by

ribonuclease, but in view of the present demonstration of the metabolic heterogeneity of microsome ribonucleoprotein their suggestion that microsome proteins are not precursors of trypsinogen or chymotrypsinogen is not necessarily valid.

Function of RNA in protein synthesis. The present work, together with other similar work already mentioned, suggests that in the normal liver cell the initial fixation of amino acids in peptide linkage takes place in a nucleoprotein fraction. Although a protein fraction with a very high rate of turnover and isolated by a variety of techniques was always associated with RNA, this does not necessarily imply that the RNA is required for protein synthesis. However, evidence has accumulated over the past 10 years that tissues with a high capacity for protein synthesis are rich in RNA (cf. Brachet, 1955), and various workers have reported that removal of RNA from whole cells or from subcellular particles by ribonuclease inhibits protein synthesis (for references see Askonas, Simkin & Work, 1956); moreover, Gale & Folkes (1955) have obtained a restoration of synthetic capacity in bacterial subcellular particles by addition of RNA or an RNA digest.

Bernhard, Gautier & Rouiller (1954) and Palade & Siekevitz (1956) have provided evidence that the liver-cell cytoplasm contains a structure, referred to as the ergastoplasm or endoplasmic reticulum, which consists of a series of convoluted membranes along which are disposed numerous small ribonucleoprotein particles. They suggested that most of the microsome fraction is derived from this structure. The present investigation demonstrates that the microsome fraction is metabolically heterogeneous. This may be accounted for, at least in part, by the heterogeneous nature of the microsome fraction itself. Kuff, Hogeboom & Dalton (1956) have emphasized that the microsome fraction may contain a variety of structures.

SUMMARY

1. Guinea pigs were killed at various time intervals after the intravenous injection of a mixture of ^{14}C -labelled amino acids (chlorella-protein hydrolysate). The liver-microsome fraction was separated into a number of subfractions of distinct composition by a procedure involving successive extraction with solutions of varying ionic strength and pH.

2. The proteins of the microsome subfractions were labelled at widely differing rates. The protein fraction with the greatest turnover rate was associated with ribonucleic acid. This protein showed a maximum activity about 30 min. after administration of labelled amino acids. Another microsome subfraction also contained ribonucleoprotein, but the protein of this fraction showed a much lower turnover rate.

3. The microsome subfractions were analysed for protein, ribonucleic acid, deoxyribonucleic acid and lipid-soluble phosphorus. Applying usual procedures for the separation of protein and nucleic acid, the ribonucleic acid of several subfractions was found to be abnormally unstable in perchloric acid. Several of the subfractions were examined in the ultracentrifuge and by electrophoresis.

4. The results have been compared with those obtained by other methods of fractionation of the microsomes, and the possible role that ribonucleic acid may play in protein synthesis is discussed.

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The Breakdown of Adenosine Triphosphate Accompanying Cholic Acid Activation by Guinea-Pig Liver Microsomes

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It has previously been shown that microsomal preparations from guinea-pig liver form cholyl-hydroxamic acid from cholic acid in the presence of adenosine triphosphate (ATP), coenzyme A (CoA) and high concentrations of hydroxylamine (Elliott, 1955, 1956a). When taurine at substrate levels is added to the same system in place of hydroxylamine, taurocholic acid is formed and this reaction is also dependent on the presence of ATP and CoA (Elliott, 1956b). Bremer (1956) and Siperstein & Murray (1956) have independently reported the synthesis of taurocholic acid by microsome preparations. These observations indicate that cholyl-CoA formation is an intermediate step in taurocholic acid synthesis. The enzyme system bringing about the synthesis of cholyl-CoA will subsequently be referred to as the cholic acid-activating system.

The present paper is concerned with the breakdown of ATP which accompanies the activation of cholic acid by the cholic acid-activating system of guinea-pig liver microsomes. It is shown that adenosine 5'-phosphate (AMP) and inorganic pyrophosphate are formed. Some properties of the microsomal pyrophosphatase which complicated the investigation are also described.

EXPERIMENTAL

Hydroxylamine. Solutions of low KCl content were prepared by the method of Beinert *et al.* (1953). Norit A was kindly supplied by Dr D. E. Hughes. The inorganic phosphate present in the charcoal was removed by boiling in *n*-HCl for 15 min. and filtering with suction. The material was washed with water until the washings were neutral and dried at 105°.