The Effect of Age on Protein Synthesis in Mouse Liver

By W. I. P. MAINWARING

Division of Chemistry and Biochemistry, Imperial Cancer Research Fund, London W.C.2

(Received 24 January 1969)

1. A system of microsomes and 105 000g supernatant from livers of old mice is less able to promote the incorporation of [^{14}C]phenylalanine into protein than a similar system from livers of young animals. 2. The decrease in [^{14}C]phenylalanine incorporation is attributable to changes in microsomes from old animals rather than in the cell-sap fraction. 3. Decreased synthetic ability is found in various classes of microsomes from older animals, namely unfractionated, light and heavy microsomes, but not in detergent-washed ribonucleoprotein particles. 4. Deletions of certain detergent-soluble microsomal proteins accompany the decreased synthetic ability of microsomes from older animals. 5. Microsomes from old mice are less responsive to a synthetic messenger RNA, polyuridylic acid, and this is partly due to a higher rate of hydrolysis in the presence of cell sap from animals of extreme age. 6. Other more direct evidence, from the priming of a cell-free protein-synthesizing system from bacteria and the examination of ribonucleoprotein particles on sucrose density gradients, suggests that senescence is accompanied by a decrease in messenger RNA content.

Some of the changes in the RNA metabolism of aging tissues were described by Mainwaring (1968) and it was speculated what effects these changes would have on protein synthesis in senescence. From the evidence of decreased nuclear RNA polymerase activity, measured in media of low ionic strength, and the decreased amount of sedimentable RNA in tissue homogenates it was suggested that the synthesis of ribosomal RNA may be particularly decreased in tissues from old animals. A further feature of aging was the decrease in the proportion of polysomes in the RNP* particulate fraction, a finding that could be explained by an age-associated depletion of m-RNA. Changes in these species of RNA could have a significant bearing on the rate of protein synthesis in tissue from old animals and a critical appraisal of this hypothesis has been the basis of the present investigation. Owing to the practical difficulty of obtaining sufficient quantities of microsomes and other subcellular fractions from mouse lung and prostate, the tissues included in the previous study (Mainwaring, 1968), the present investigation was necessarily restricted to a study of protein synthesis in livers of aging animals only. This was not a prohibitive restriction because the changes in the RNA metabolism of several tissues in aging animals were remarkably similar, suggesting that the

findings from liver could be justifiably applied to other tissues. Several reports have appeared on the changes in RNA metabolism and protein synthesis during the development and maturation of liver (Oliver, Blumer & Witham, 1963; Murthy, 1966), but little work has been published on protein synthesis in liver of aged animals. Nevertheless it is believed that protein synthesis is impaired in senescence (see, e.g., review by Medvedev, 1966), although the precise biochemical explanation of this change has not been found. Particular attention has been given in the present work to the activities of liver microsome and RNP-particle preparations from animals of different ages, a comparison of the pH 5 enzyme activities with age and a study of m-RNA content of liver during aging of the animals.

MATERIALS AND METHODS

Chemicals. Chemicals, A.R. grade, were generally used without further purification. However, diphenylamine and orcinol were recrystallized from 70% (v/v) ethanol and benzene respectively before use. Phenol was redistilled and the fraction boiling at 180–182° was stored frozen at –15°. Glass-distilled water was used exclusively in making up all solutions. RNA was isolated from baker's yeast as directed by Crestfield, Smith & Allen (1955). Crystalline bovine serum albumin, sodium dodecyl sulphate, polyU (ammonium salt, type 1), phosphoenolpyruvate (monopotassium salt), ATP, GTP and tris base (99.5% pure) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6.

Bioch. 1969, 113

* Abbreviations: RNP, ribonucleoprotein; polyU, polyuridylic acid; m-RNA, messenger RNA; t-RNA, transfer RNA.

Solutions of polyU, phosphoenolpyruvate, ATP and GTP were adjusted to pH 7.8 with tris base before they were made up to volume, and 0.5 ml. portions were stored frozen in small Perspex tubes. Amino acids (pure L-isomers wherever possible) and bentonite were purchased from British Drug Houses Ltd., Poole, Dorset. *Escherichia coli* B cells were generously provided by Dr K. Sargeant, Microbiological Research Establishment, Porton, Wilts., and stored at -15° . Alumina grinding powder (Griffin and George Ltd., London W.5; grade 3/50) was washed with water and dried at 60° before use. L-[U- ^{14}C]Phenylalanine (459 mc/m-mole) was supplied by The Radiochemical Centre, Amersham, Bucks. Pyruvate kinase was obtained from Boehringer (London) Corp., London W.5. Triton X-100 was a gift from Lennig Chemicals Ltd., London W.C.1.

Chemical analyses. Protein determinations were performed by the Folin-Ciocalteu procedure (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as standard. Samples for RNA analysis were precipitated in the cold in 0.4 M-HClO₄, washed twice in cold 0.2 M-HClO₄ and extracted with 1.0 ml. of 0.5 M-HClO₄ at 70° for 20 min. After centrifugation, the supernatant was analysed for ribonucleotides by the method of Brown (1946), with yeast RNA as standard.

Counting of radioactivity. ^{14}C was counted in a Packard Tri-Carb model 3003 scintillation spectrometer at an efficiency of 40%. Quenching was determined by the channels-ratio method (Baillie, 1960). The phosphor contained 5 g. of 2,5-diphenyloxazole and 0.3 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene dissolved in 1 l. of toluene.

Animals. These were male C57 black-and-tan mice, segregated according to their month of birth. Randomly selected mice within a given age group were killed by cervical dislocation and the livers were rapidly removed and rinsed twice in the appropriate ice-cold medium before the isolation of subcellular fractions.

Isolation of subcellular particles. All isolation procedures were conducted as near 0° as possible. The livers of two mice were pooled and minced with scissors. The tissue was then homogenized in a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle. The pestle speed was approx. 1000 rev./min. and at least six complete up-and-down movements of the pestle were performed for each homogenization. The pestle was 2.0 cm. in diameter and the clearance between this and the mortar was 0.20–0.24 mm.

(a) Microsomes. The tissue was homogenized in 13.5 ml. of medium A (0.15 M-sucrose, 10 mM-MgCl₂, 25 mM-KCl, 35 mM-tris base) adjusted to pH 7.8 with 1 M-HCl, with a glass electrode at 25° (Rendi & Hultin, 1960). After centrifugation at 15 000 g for 10 min., the supernatant was centrifuged at 105 000 g for 50 min. at 4° , in the swing-out S40 rotor of a Christ Omega ultracentrifuge. Samples of clear supernatant (cell sap) were carefully aspirated, avoiding the floating layer of fat, and kept at 0° as a source of activating enzymes and t-RNA. For certain experiments, samples (2.0 ml.) of cell sap were purified by passage through a column of Sephadex G-25 (1.2 cm. $\times$ 20 cm.), equilibrated with medium A, to remove low-molecular-weight inhibitors (Mansbridge & Korner, 1963). The pellet of microsomes was gently resuspended in 15 ml. of medium A by gentle hand homogenization, followed by centrifugation at 105 000 g for 35 min. The washed microsomes were dispersed in 2.0 ml. of medium A, in a loose-fitting TenBroeck homogenizer.

Subfractionation of microsomes into 'heavy' and 'light' fractions was accomplished essentially as described by Campbell, Serck-Hanssen & Lowe (1965). Both microsome fractions were individually resuspended in 2.0 ml. of medium A. The final clear supernatant was cell sap and samples were kept for incorporation experiments.

(b) RNP particles. Pooled livers from two animals were homogenized in 10 ml. of 35 mM-tris-HCl buffer, pH 7.8, containing 0.35 M-sucrose, 25 mM-KCl and 10 mM-MgCl₂. RNP particles were isolated by the procedure of Hunter & Korner (1966). The particles were gently resuspended in a total volume of 2.0 ml. of medium A. In preliminary experiments RNP particles were freed of remaining traces of detergent by further centrifugation through medium A. This practice was discontinued when it was found that the small amount of residual detergent decreased subsequent measurements of protein synthesis *in vitro* by only 5%.

(c) Nuclei. Tissue was minced with curved scissors and homogenized in 20 ml. of 0.25 M-sucrose containing 3 mM-CaCl₂. After filtration through 100-gauge stainless-steel grids, a crude nuclear pellet was obtained by centrifugation at 700 g for 10 min. After one wash in sucrose-CaCl₂ solution, two approaches were used for further purification of the nuclei. In the first method, the crude 700 g pellet was evenly resuspended in 20 ml. of 2.0 M-sucrose containing 1 mM-CaCl₂ and the nuclei were collected by centrifugation at 30 000 g for 1 hr. In the second procedure, the crude nuclei were washed three times in 0.25 M-sucrose containing 0.5% (v/v) Triton X-100 and collected by centrifugation at 700 g (Hymer & Kuff, 1964).

Preparation of a preincubated S-30 fraction. Freshly thawed *E. coli* cells were disrupted by grinding in a mortar with twice their weight of washed grinding alumina. A preincubated S-30 preparation was prepared as described by Nirenberg (1964) and charged with L-amino acids, including phenylalanine. The dialysed preparation was quickly frozen in a solid CO₂-acetone freezing-mixture and stored at -15° . Stored thus, the preparations were stable for at least 1 month.

Washed *E. coli* ribosomes were also prepared according to the procedure of Nirenberg (1964), gently resuspended in 10 mM-tris-HCl buffer, pH 7.8, containing 14 mM-magnesium acetate and 60 mM-KCl, and stored at -15° .

Extraction of RNA. Several potential methods for the extraction of nuclear RNA were tested, but that of Georgiev (1967) proved to be the most reproducible. Microsomal (cytoplasmic) RNA was isolated as described by di Giralamo, Henshaw & Hiatt (1964).

Assay of protein synthesis. (a) Microsomes. The following basic components were mixed in tubes supported in an ice bath: 0.20 ml. of cell sap, 0.2 μC of [^{14}C]phenylalanine, 2 μmoles of ATP, 7.5 μmoles of phosphoenolpyruvate and 50 μg . of pyruvate kinase. In certain cases 50 μg . of polyU was added and in one series of experiments 0.5 μmole of GTP and 2 μmoles of β -mercaptoethanol were also included. Samples of microsome suspension, i.e. 0.20 ml. (250–400 μg . of RNA) of total, heavy or light microsomes, were added and the total volume was adjusted to 1.0 ml. with 10 mM-tris-HCl buffer, pH 7.8, and finally enough 0.1 M-MgCl₂ was added to give an overall concentration of 10 mM with respect to MgCl₂. Incubation was in air for 45 min. at 37° , together with blanks from which microsomes were omitted. The reactions were halted by the addition of 3 ml. of 5% (w/v) trichloroacetic acid, followed by 10 min. standing in

ice. The acid-insoluble residue was collected by centrifugation, resuspended in 3 ml. of 5% trichloroacetic acid, heated in a boiling-water bath for 15 min. and compacted by further centrifugation in the cold. After gentle dispersion in 3 ml. of 5% trichloroacetic acid with a Pasteur pipette the precipitates were transferred, with washings, on to pre-soaked Millipore filters (code no. PHWP02500). The membranes were washed three times with 5 ml. of 5% trichloroacetic acid, placed in 10 ml. of phosphor and counted in a scintillation spectrometer. Treatment of the acid-insoluble residues with organic solvents did not decrease the bound radioactivity further, suggesting that washing with non-aqueous solvents was not necessary. Counts were corrected for blanks and background, and the incorporation of radioactivity was calculated in terms of pmoles of [^{14}C]-phenylalanine/250 μg . of microsomal RNA. The conversion of d.p.m. into pmoles of [^{14}C]-phenylalanine was made from a standard curve. Under the conditions of counting employed, the incorporation of 1 pmole of [^{14}C]-phenylalanine was equivalent to 1300 d.p.m.

(b) RNP particles. The assay system contained the following components, mixed on ice: 0.20 ml. of cell sap, 0.2 μC of [^{14}C]-phenylalanine, 3 μmoles of ATP, 10 μmoles of phosphoenolpyruvate, 50 μg . of pyruvate kinase, 100 μg . of polyU where appropriate, and 0.20 ml. of RNP particle suspension (300–600 μg . of RNA). The volume was made up to 1.0 ml. with 10 mM-tris-HCl buffer, pH 7.8, and sufficient 1.0 M stock salt solutions were added to make the system 10 mM, 40 mM and 100 mM overall with respect to MgCl_2 , NaCl and KCl respectively (Munro, Jackson & Korner, 1964). The reactions were allowed to proceed for 45 min. at 37°, together with blanks from which RNP particles were omitted, and then stopped by the addition of 3 ml. of 5% (w/v) trichloroacetic acid. The subsequent treatment of the radioactive precipitates was conducted as described for microsomes.

Measurement of m-RNA by using the S-30 system. The assay of m-RNA was performed essentially as described by Nirenberg (1964), except that non-radioactive phenylalanine was omitted from mixture II. By using 150 μl . of S-30 preparation (910 μg . of protein, 305 μg . of RNA) and [^{14}C]-phenylalanine, the system was saturated with 15 μg . of polyU. Liver RNA fractions, prepared as described above, were added in amounts up to 100 μg ./assay in both the presence and the absence of 5 mM-ammonium acetate (di Giralamo *et al.* 1964). The incubation period was extended to 45 min. at 37° with the mammalian m-RNA samples.

Assay of pH 5 enzyme activities. In testing the activity of t-RNA and activating enzymes, it seemed desirable to employ pH 5 enzyme fractions rather than cell sap directly, for in this way possible variations in soluble activating factors such as GTP and also soluble inhibitors (Mansbridge & Korner, 1963) would be precluded. Accordingly, 5.0 ml. of clear cell sap was adjusted to pH 5.2 by the dropwise addition of 0.2 M-acetic acid, and the precipitate, termed the 'pH 5 enzyme fraction', was collected by centrifugation at 2000g and resuspended in 2.5 ml. of 10 mM-tris-HCl buffer, pH 7.8, containing 10 mM-magnesium acetate. The fraction was rendered completely soluble by readjustment of the pH to 7.8 by the addition of tris base. The assay was essentially based on that of Nirenberg (1964) for *E. coli* S-30 preparations, but with the heterologous liver pH 5 enzyme fraction replacing the homologous *E. coli* supernatant fraction. The use of a mixed system is amply

justified by recent work demonstrating the almost complete universality of the genetic code and that heterologous cell-sap and ribosome fractions work efficiently together (e.g., Ehrenstein & Lipmann, 1961). The components in the assay system were mixed in the following order in tubes cooled in ice: 75 μl . of mixed solution II (Nirenberg, 1964), 50 μl . of washed *E. coli* ribosomes (250 μg . of RNA), 20 μl . of polyU (20 μg .), 25 μl . (0.2 μC) of L-[^{14}C]-phenylalanine and 25–100 μl . of liver pH 5 enzyme fraction (80–320 μg . of protein) and water to total volume 0.27 ml. After incubation of the samples at 37° for 30 min., the labelled protein was collected and washed as described for liver microsomes. The relationship between the amounts of pH 5 enzyme fraction added and of [^{14}C]-phenylalanine incorporated was not perfectly linear and the activity was calculated from the mean of assays performed at two different enzyme concentrations.

Electrophoresis of Triton X-100-soluble microsomal proteins. During this investigation the possibility that these proteins might determine the rate of protein synthesis in microsomal preparations became apparent and it seemed desirable to develop methods for isolating and characterizing these Triton-soluble proteins. Microsome pellets were resuspended in 0.90 ml. of medium A and 0.10 ml. of 10% (w/v) Triton X-100 in 50 mM-tris-HCl buffer, pH 7.8. After gentle but thorough mixing the suspension was centrifuged at 105000g for 90 min. in cellulose nitrate tubes supported within Teflon holders in the Ti-50 rotor of a Spinco ultracentrifuge. The clear supernatants were made 5 M with respect to urea and stored at 0–4°. Resolution of the complex protein mixture was done by electrophoresis in 7.5% (w/v) polyacrylamide gels. The method was based essentially on that of Takayama, MacLennan, Tzagoloff & Stoner (1964). The gels were mounted in electrophoresis apparatus (Shandon Scientific Co. Ltd., London N.W.10) and the reservoirs were filled with 10% (v/v) acetic acid. Samples (approx. 50 μl .; 150–200 μg . of protein) were applied to the gels and a constant current of 4 mA/gel was passed for 90–120 min. at room temperature, with the cathode connected to the lower reservoir. The migration of a sample of Pyronin Y (1 mg./100 ml.) served to identify the movement of the ion front. After being stained overnight in 1% (w/v) Naphthalene Black in methanol–10% acetic acid (1:1, v/v), the gels were destained electrophoretically in 10% acetic acid at 4 mA/gel. This procedure provided a satisfactory separation of basic microsomal proteins. Acidic microsomal proteins were separated by a similar procedure, but the buffer was 50 mM-tris-acetate, pH 8.3, and the polarity of the electrodes during electrophoresis was reversed.

Separation of bound and free ribosomes in microsomal preparations. Such separations were performed by sucrose-density-gradient centrifugation. The method was essentially that of Henshaw, Bojarski & Hiatt (1963), but scaled down to gradients of 4.0 ml. total volume. After centrifugation at 75000g the tubes were punctured with a 28 G hypodermic needle and 3-drop fractions were collected, and the E_{260} was monitored.

RESULTS

Comparison of the incorporation of [^{14}C]-phenylalanine into protein by unfractionated liver microsomes from rats of different ages. Before embarking on these comparative studies, it was first shown

Table 1. Comparison of the activities of liver microsomes from mice of various ages for the incorporation of [^{14}C]phenylalanine

Methods for the preparation of the microsomes and the basic components of the assay system are described in the Materials and Methods section. Microsomes were prepared from livers pooled from two animals, and were incubated with cell sap from that age group. Incorporation is expressed as mean ($\pm$ s.d.) values, in pmoles of [^{14}C]phenylalanine incorporated/250 μg . of microsomal RNA/45 min. incubation at 37°. At least three experiments were performed in each age group.

	Age group			<i>P</i> (group I versus group III)
	I (5 months)	II (17.5 months)	III (30 months)	
(a) RNA/protein ratio of microsomes	0.32 $\pm$ 0.01	0.25 $\pm$ 0.01	0.20 $\pm$ 0.04	—
(b) Incorporation of [^{14}C]phenylalanine				
PolyU absent	3.85 $\pm$ 0.09	2.95 $\pm$ 0.25	1.80 $\pm$ 0.35	< 0.01
PolyU (50 μg .) present	7.50 $\pm$ 0.04	5.02 $\pm$ 0.10	2.80 $\pm$ 0.25	< 0.01
Ratio of activity polyU present/ polyU absent	1.95	1.70	1.55	—

that the amount of [^{14}C]phenylalanine incorporated was directly proportional to the amount of microsomal suspension added to the test system; hence the amount of microsomes was the only limiting factor. Secondly, with regard to stimulation of the system by polyU, it was shown that maximum incorporation of [^{14}C]phenylalanine occurred at an overall magnesium chloride concentration of 10 mM, as found by Campbell, Cooper & Hicks (1964) for unfractionated microsomes from rat liver. The results of experiments on the incorporation of [^{14}C]phenylalanine into protein in microsomes of various ages are summarized in Table 1. In these experiments an attempt was made to simulate conditions existing *in vivo* as closely as possible. Mercaptoethanol, for example, was intentionally omitted from the assay system because Oeriu (1964) has suggested that oxidation of thiol groups may be an important feature of the aging process. Thus the addition of mercaptoethanol could offset the genuine deleterious changes in the proteins in the cell sap from old animals and mask a decrease in protein synthesis. It was found that the incorporation of [^{14}C]phenylalanine was dependent on the age of the animals from which the microsomes were prepared and that the microsomes from the oldest animals were least able to promote protein synthesis. The microsomes from old animals also contained a lower RNA/protein ratio and showed the least response to the addition of polyU. The latter effect can be attributed either to excessive degradation of polyU in the presence of cell fractions from liver of old animals or to a higher endogenous content of m-RNA in the microsomes from old animals. This particular problem is examined more closely below. Certainly the decrease in protein synthesis with age cannot be attributed simply to differences in the stability of the microsomes during incubation, because the time-course of the incorporation of [^{14}C]phenyl-

Fig. 1. Time-course of the incorporation of [^{14}C]phenylalanine into protein with liver microsomes from young (5-month-old) animals ($\square$) and aged (30-month-old) animals (Δ). Incorporation is expressed as pmoles of [^{14}C]phenylalanine/250 μg . of microsomal RNA.

alanine into microsomes from animals of different ages was identical (Fig. 1).

Effect of interchanging components from different age groups on protein synthesis in vitro. On the evidence presented above alone it was impossible to decide whether the decrease in the rate of [^{14}C]phenylalanine incorporation was due to age-associated changes in the microsomes or the cell sap. To clarify this point, cell sap and microsomes were interchanged and assayed for protein synthesis. In addition, the effect of removing low-molecular-weight inhibitors from the cell sap (Mansbridge & Korner, 1963) was also investigated. In contrast with previous experiments, β -mercaptoethanol was included in the assay medium and also GTP. From the summary of the results given in Table 2 it is clear that the decreased incorporation of [^{14}C]phenylalanine into protein in old animals is almost

Table 2. *Effect of homologous and heterologous cell sap in the incorporation of [¹⁴C]phenylalanine into protein of microsomes of different ages*

'Young' and 'old' refer to animals 5 and 30 months old respectively. Microsomes were resuspended such that RNA concentrations were identical, and 0.20 ml. of suspension was incubated with 0.20 ml. of cell sap from the same (homologous) and different (heterologous) homogenates. GTP (0.5 μ mole) and β -mercaptoethanol (2.0 μ moles) were included in the assay medium. Activity is expressed as pmoles of [¹⁴C]phenylalanine incorporated/250 μ g. of microsomal RNA/45 min. incubation at 37°.

Source of cell sap		Source of microsomes	
		(I) Young	(II) Old
(I) Young	(i) untreated	10.45	5.85
	(ii) after gel filtration	12.45	8.00
(II) Old	(i) untreated	10.00	5.80
	(ii) after gel filtration	11.95	8.05
Ratio of activity old/young microsomes with		(a) old cell sap	0.56
		(b) young cell sap	0.58
Effect of gel filtration on cell sap (compared with		(a) young system	1.19
untreated sap in a homologous system) in		(b) old system	1.37

Table 3. *Incorporation of [¹⁴C]phenylalanine into protein with fractionated liver microsomes from animals of different ages*

The method of preparing the microsome fractions is described in the Materials and Methods section. Cell fractions were prepared from livers pooled from two animals. Microsomes were incubated with homologous cell sap, i.e. from the same homogenate. Incorporation is expressed as pmoles of [¹⁴C]phenylalanine incorporated/250 μ g. of microsomal RNA/45 min. incubation at 37°.

	Age group		
	I (5 months)	II (17.5 months)	III (30 months)
(a) RNA/protein ratio of microsomes			
(1) Heavy-microsome fraction	0.31	0.31	0.34
(2) Light-microsome fraction	0.52	0.43	0.34
(b) Incorporation of [¹⁴ C]phenylalanine			
(1) Heavy-microsome fraction			
(i) PolyU absent	1.25	0.75	0.80
(ii) PolyU (50 μ g.) present	1.70	1.70	2.30
(iii) Ratio of activity polyU present/polyU absent	1.36	2.27	3.47
(2) Light-microsome fraction			
(i) PolyU absent	3.20	2.35	0.95
(ii) PolyU (50 μ g.) present	6.35	4.20	3.10
(iii) Ratio of activity polyU present/polyU absent	1.97	1.79	3.26

exclusively due to changes in the microsomes alone, for, irrespective of the source of cell sap, microsomes from old animals were always less efficient in promoting protein synthesis. Similarly, microsomes from young animals were not inhibited by cell sap from old animals. In complete accord with this finding, treatment of the cell-sap fractions with Sephadex G-25 resulted in a similar increase in activity in systems from both old and young mice, suggesting that an age-associated increase in the concentration of low-molecular-weight inhibitors is not a plausible explanation of the results. The inclusion of GTP and β -mercaptoethanol was uniformly beneficial to systems from both old and

young mice. In later experiments, however, microsomes were always incubated with homologous cell sap.

Studies on the incorporation of [¹⁴C]phenylalanine into protein with fractionated liver microsomes. Studies were made on the activities of the 'heavy'-microsome and 'light'-microsome fractions of liver from animals of different ages. The chemical composition of the two fractions and their response to the addition of polyU are shown in Table 3. Aging was again accompanied by a decrease in the incorporation of [¹⁴C]phenylalanine into protein in both the heavy-microsome and light-microsome fractions. The activity of the heavy-microsome

Table 4. *Comparison of the activities of liver RNP particles from mice of different ages for the incorporation of [¹⁴C]phenylalanine*

Methods for the preparation of the RNP particles and the components in the assay system are given in the Materials and Methods section. RNP particles were prepared from livers pooled from two animals, and incubated with cell sap from that age group. Incorporation is expressed as mean ($\pm$ s.d.) values, in pmoles of [¹⁴C]phenylalanine incorporated/250 μ g. of ribosomal RNA/45 min. incubation at 37°, with three experiments in each age group.

	Age group		
	I (5 months)	II (17.5 months)	III (30 months)
(a) RNA/protein ratio of RNP particles	1.07	1.13	1.21
(b) Incorporation of [¹⁴ C]phenylalanine			
(i) PolyU absent	3.20 $\pm$ 0.07	2.90 $\pm$ 0.13	2.85 $\pm$ 0.12
(ii) PolyU (100 μ g.) present	6.15 $\pm$ 0.09	5.40 $\pm$ 0.21	3.70 $\pm$ 0.12
(iii) Ratio of activity polyU present/polyU absent	1.95	1.86	1.30

fraction was lower than that of unfractionated microsomes, but the light-microsome fraction was of similar activity to the unfractionated microsomes. Similarly, the heavy-microsome fraction and the unfractionated microsomes had a similar chemical composition, whereas the light-microsome fraction had a higher RNA/protein ratio. It should be stressed, however, that the differences between the heavy-microsome and light-microsome fractions tended to disappear in the aged (30-month-old) group. The effect of adding polyU to the two microsome subfractions was different from the response found in unfractionated microsomes. It was found that polyU had the least effect on the microsomes from young animals, but both types of microsomes from aged animals were strongly stimulated. Overall, the principal lesion occurring with increasing age is found in the light-microsome fraction. The yields of light microsomes and heavy microsomes from 30-month-old animals were exceedingly variable, and this precluded a meaningful comparison of the proportions of the two classes of microsomes with increasing age.

Protein synthesis in liver RNP particles from animals of different ages. When the activities of liver RNP particles in stimulating the incorporation of [¹⁴C]phenylalanine into protein were compared, a uniformity with respect to both chemical composition and synthetic ability was revealed (Table 4), at least in the absence of polyU. The RNA/protein ratio approximated to that reported by Campbell *et al.* (1964) for rat liver RNP preparations. This uniformity in the rate of [¹⁴C]phenylalanine incorporation was in complete contrast with the results obtained with the liver microsome preparations. These findings point to a unique control of protein synthesis by components in the endoplasmic reticulum that are rendered soluble by treatment with detergents. Liver RNP particles from old animals were less stimulated by polyU than RNP particles from young animals.

Nature of the Triton-soluble microsomal proteins. The Triton-soluble microsomal proteins gave a complex series of bands after electrophoresis in polyacrylamide gels (Plate 1). The basic proteins gave a large number of well separated bands, some of which stained more intensely than others. With increasing age of the animals one very prominent, fast-moving, band virtually disappeared and the pattern of slower-moving bands became more complex. The acidic proteins were not so well resolved, but again two fast-moving bands were absent from preparations of liver microsomes from old animals. The deletion of all these bands was reproducible through several experiments. Since a decrease in the synthetic ability of the microsomes follows the deletion of these bands, it is tempting to suggest that aging is accompanied by a loss of proteins within the endoplasmic reticulum that serve as activators of protein synthesis.

Aging and the activity of liver pH5 enzyme fraction. On the evidence presented so far, the decrease in protein synthesis with age cannot be attributed to changes in the activity of the cell-sap fraction. This point was examined more precisely in an assay system where the content of activating enzymes and t-RNA species, in the form of a pH5 enzyme fraction, was limiting (see Fig. 2). The use of bacterial ribosomes was prompted by their stability during storage and their high activity in polyphenylalanine synthesis in the presence of saturating amounts of polyU. In this system the pH5 enzyme fractions from animals of different ages were of identical activity, suggesting that even if the concentration of non-sedimentable RNA may increase with age (Mainwaring, 1968) this is not commensurate with an increase in the amount of t-RNA species, or at least of phenylalanine t-RNA.

Concentrations of m-RNA in livers of aged animals. Considerable use has been made in the past of synthetic polyribonucleotides with messenger-like characteristics to establish, somewhat indirectly,

EXPLANATION OF PLATE I

Separation of Triton-soluble microsomal proteins in 7.5% (w/v) polyacrylamide gels; (a) basic proteins separated at pH 4.0; (b) acidic proteins separated at pH 8.3. Gels 1, 2, and 3 show the proteins from livers of animals 5, 14 and 30 months old respectively.

Fig. 2. Relationship between the amount of liver pH5 enzyme fraction and the incorporation of $[^{14}\text{C}]$ phenylalanine into protein, in a cell-free system. Details of the assay system are given in the Materials and Methods section.

Fig. 3. Effect of incubating polyU with cell-sap fractions on its ability to prime the *E. coli* S-30 system. PolyU (100 μg.) was incubated with 0.25 ml. of cell sap from animals of different ages, containing the same amount of protein, for 15 min. at 37°. The polyU was extracted into phenol, (as for cytoplasmic RNA; see the Materials and Methods section) and introduced into the assay system. ○, Untreated polyU; △, cell sap from young (5-month-old) mice; □, cell sap from old (30-month-old) mice. Amounts of polyU were calculated from the extinction at 260 nm., where 20 μg. of untreated polyU $\equiv E_{260}^{1\text{cm.}} \cdot 0.545$.

the concentrations of endogenous m-RNA in microsome and RNP preparations, including those from liver (Maxwell, 1962). Attempts to stimulate liver microsome and RNP preparations of different ages did not produce clear-cut results. In the main, the cytoplasmic particles from old animals were less responsive to added polyU. In comparative studies it is necessary to assume that the synthetic m-RNA is equally stable under the conditions of assay with different preparations. However, Matthaei, Jones, Martin & Nirenberg (1962) showed that the priming efficiency of polyU in cell-free systems was a

Fig. 4. Effect of the method of purification and the presence of NH_4^+ ions on the stimulation of *E. coli* S-30 system by liver nuclear RNA. For details of preparation of nuclei and RNA extraction see the Materials and Methods section. Assays were performed essentially as described by Nirenberg (1964). Incorporation of $[^{14}\text{C}]$ phenylalanine into protein is quoted as c.p.m. in the presence of: ○, polyU; △ and ▲, RNA from nuclei purified by treatment with Triton X-100; □ and ■, RNA from nuclei purified by centrifugation in 2M-sucrose. △ and □, 5 mM-Ammonium acetate present; ▲ and ■, ammonium acetate absent.

function of chain length and that short chains of polymer were effective inhibitors. This phenomenon is not shown with all synthetic m-RNA species, e.g. polyadenylic acid (Jones, Townsend, Sober & Heppel, 1964). Nevertheless this was important with the present study with polyU, and, as illustrated in Fig. 3 incubation of polyU with cell sap from young and old animals resulted in a decrease in priming of $[^{14}\text{C}]$ phenylalanine incorporation in the *E. coli* S-30 system, especially with the old animals. The reason why polyU is less efficient in stimulating protein synthesis in microsomes from old animals may thus be due to the higher rate of degradation of polyU. The conflicting results obtained with light-microsome fractions may be explained by the higher proportion of free ribosomes in these fractions (Campbell *et al.* 1965), which, in binding m-RNA more avidly, may afford it protection from enzymic degradation.

It is generally agreed that the best practical test for m-RNA is its ability to prime protein synthesis in a cell-free system of ribosome monomers. The most widely used system is the preincubated S-30 system (Nirenberg & Matthaei, 1961). Despite the use of several methods for isolating both nuclear and cytoplasmic RNA from mouse liver (di

Giralamo *et al.* 1964; Barondes, Dingman & Sporn, 1962; Georgiev, 1967) the stimulation of the S-30 system by liver RNA was never very marked. Indeed, the incorporation of [^{14}C]phenylalanine was only detectable when non-radioactive phenylalanine was omitted from the assay system. Cytoplasmic RNA barely stimulated the cell-free system, irrespective of the method of preparation and assay, but nuclear RNA, especially when isolated from Triton-washed nuclei, did stimulate the system to a limited extent (Fig. 4). The addition of ammonium acetate was beneficial to the system, in agreement with the results of di Giralamo *et al.* (1964). The amount of incorporation was very much lower than that reported by Barondes *et al.* (1962) and Bondy

& Roberts (1967) with mammalian m-RNA samples, and at the point where polyU saturated the system the percentage of radioactivity incorporated in the presence of liver RNA was less than 1%. This extent of incorporation was more in agreement with results of Lang & Sekeris (1964), who used a homologous assay system from liver. Despite the low extent of incorporation the template activities of liver nuclear RNA from old and young mice were compared (Fig. 5). Such experiments suggest that nuclei from old animals contain less m-RNA than nuclei from young animals.

Ratio of bound to free ribosomes in livers of aged animals. In view of the problems encountered with the other methods of determining m-RNA, the examination of the liver ribosome population was undertaken. In the experimental system of Henshaw *et al.* (1963) bound ribosomes are separated from the free ribosomes, which contain little, if any, m-RNA. As shown in Fig. 6, aging is accompanied by a significant rise in the proportion of free ribosomes. Based on the areas demarcated on Fig. 6, the ratio of bound to free ribosomes was 5.0, 3.56 and 1.74 in animals 5, 14 and 30 months old respectively. The profile for the young animals is similar to that obtained by Henshaw *et al.* (1963) for rat liver. This result could suggest that there are fewer membrane-bound ribosomes in old animals. In any event, these changes are the reverse of the situation found during liver maturation (Oliver *et al.* 1963).

Fig. 5. Comparison of the priming of the *E. coli* S-30 system by nuclear RNA from liver of young animals (5-month-old) (□) and old animals (30-month-old) (Δ). Quenching was the same in all samples and incorporation of [^{14}C]phenylalanine into protein is quoted as c.p.m.

DISCUSSION

The evidence presented suggests that liver of older mice is less able to promote protein synthesis. Further, this decreased protein synthesis cannot be

Fig. 6. Separation of bound and free ribosomes from liver microsomes from mice aged (a) 5 months, (b) 14 months and (c) 30 months. Gradients were made essentially as described by Henshaw *et al.* (1963), with sedimentation from right to left. Arrows mark the interface between 50 and 20% (w/v) sucrose layers. The areas B and F, delineated by the broken lines, were determined by the 'counting of squares' method and used to calculate the ratio of bound to free ribosomes, i.e. the B/F area ratio.

due to changes in the stability of the microsomes from old animals *in vitro* (McQuorquodale, Veach & Mueller, 1960). This is in agreement with the reported decrease in serum albumin synthesis in aged rats (Kornienko, 1960, quoted by Medvedev, 1966), for this protein is a specific product of protein synthesis in liver (Sargent & Campbell, 1965). No substantial evidence could be found to suggest that the decrease in protein synthesis was due to age-associated changes in the cell-sap fraction. Similar conclusions may be drawn from studies with pH 5 enzyme fractions. Thus, despite the increase in amount of non-sedimentable RNA with age (Mainwaring, 1968), this is not associated with any significant change in the biological activity of this fraction, at least with respect to t-RNA. Further, changes in low-molecular-weight factors in the cell sap cannot be considered a major event in the aging process. This would apply both to inhibitors (Mansbridge & Korner, 1963) and to activators, such as GTP, including its effect on the microsomal inhibitor of amino acid incorporation postulated by Hoagland, Scornik & Pfefferkorn (1964).

The decrease in protein synthesis was found in experiments with all classes of microsomes from old animals, namely unfractionated, light and heavy fractions. Throughout this study the incorporation of radioactive amino acid into protein has been referred to a standard amount of RNA, but there was a similar decrease with respect to microsomal protein. The main synthetic activity in mouse liver appeared in the light-microsome fraction, which Campbell *et al.* (1965) suggest contains a high proportion of membrane-free polysomes. It may be argued that the higher rate of incorporation of amino acids into protein in young animals is due to a higher proportion of polysomes. This concept is substantiated by previous evidence on the RNP populations of aged liver (Mainwaring, 1968) and the present finding that aged cells contain a higher proportion of free ribosomes or ribosomes deficient in m-RNA. All these techniques, however, suffer from the possible degradation of m-RNA during either isolation or subsequent incubation. This may be particularly true in liver of aging animals, which is characterized by an increase in ribonuclease activity (Mainwaring, 1968), and this may well explain the particular susceptibility of polyU to incubation with cell sap from old animals. Break-down of m-RNA during amino acid incorporation into proteins has been described by Tissières & Watson (1962). It may well be, however, that this ready degradation of m-RNA is a characteristic feature of older cells and not just an experimental artifact. Certainly the work of Danon & Cividalli (1968) on the maturation of reticulocytes, which is an aging process, is particularly germane to this

discussion. They have found that the progressive decrease in protein synthesis during erythrocyte maturation is almost certainly due to polysome depletion and this they explained by either a diminished ability of m-RNA to bind the ribosome monomers together or to a decrease in the length of the m-RNA as it reaches the end of its 'life span'. This seems the most satisfactory explanation overall, because the results of priming the *E. coli* S-30 system with liver nuclear RNA were not sufficiently different to suggest that aging is accompanied by a decrease in m-RNA synthesis. With regard to the low extent of incorporation in this heterologous system, there now seems to be reasonable evidence that the initiation of protein synthesis in mammalian systems does not involve *N*-formylmethionyl-t-RNA (Arnstein & Rahaminoff, 1968), which Capecchi (1966) suggested was involved in bacterial protein initiation. It may be that liver m-RNA cannot present the bacterial system with the most appropriate initiating codon.

Similar incorporation of [¹⁴C]phenylalanine into protein with RNP preparations of different ages inevitably focuses attention on the way in which membrane proteins can control the rate of protein synthesis. From experiments with regenerating liver Tsukada & Lieberman (1965) consider that phospholipids facilitate the formation of polysomes, whereas Campbell, Lowe & Serck-Hanssen (1967), although finding no evidence for an increase in the polysome content of regenerating liver, reaffirm the importance of membrane proteins in controlling protein synthesis. The difference in the electrophoretic patterns of the Triton-soluble microsomal proteins probably reflect a major cytoplasmic lesion during senescence, for other workers have shown that the nucleotide composition of liver ribosomal RNA remains constant with increasing age (Nikitin & Shereshevskaya, 1962).

The author thanks Dr L. M. Franks for his interest and the supply of animals, Dr D. A. Woods for ultracentrifuge facilities and Mrs Brenda Peterken and Mr L. Brann for technical assistance.

REFERENCES

- Arnstein, H. R. V. & Rahaminoff, H. (1968). *Nature, Lond.*, **219**, 942.
Baillie, L. A. (1960). *Int. J. appl. Radiat. Isotopes*, **8**, 1.
Barondes, S. H., Dingman, C. W. & Sporn, M. B. (1962). *Nature, Lond.*, **196**, 145.
Bondy, S. C. & Roberts, S. (1967). *Biochem. J.* **105**, 1111.
Brown, A. H. (1946). *Arch. Biochem.* **11**, 269.
Campbell, P. N., Cooper, C. & Hicks, M. (1964). *Biochem. J.* **92**, 225.
Campbell, P. N., Lowe, E. & Serck-Hanssen, G. (1967). *Biochem. J.* **103**, 280.
Campbell, P. N., Serck-Hanssen, G. & Lowe, E. (1965). *Biochem. J.* **97**, 422.

- Capecchi, M. R. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 1517.
- Crestfield, A. M., Smith, K. C. & Allen, F. W. (1955). *J. biol. Chem.* **216**, 185.
- Danon, D. & Cividalli, L. (1968). *Biochem. biophys. Res. Commun.* **30**, 717.
- di Giralamo, A., Henshaw, E. C. & Hiatt, H. H. (1964). *J. molec. Biol.* **8**, 479.
- Ehrenstein, G. von & Lipmann, F. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 941.
- Georgiev, G. (1967). *Progr. Nucleic Acid Res.* **6**, 259.
- Henshaw, E. C., Bojarski, T. B. & Hiatt, H. H. (1963). *J. molec. Biol.* **7**, 122.
- Hoagland, M. B., Scornik, O. A. & Pfefferkorn, L. C. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 1184.
- Hunter, A. R. & Korner, A. (1966). *Biochem. J.* **100**, 73P.
- Hymer, W. C. & Kuff, E. L. (1964). *J. Histochem. Cytochem.* **12**, 359.
- Jones, O. W., Townsend, E. E., Sober, H. A. & Heppel, L. A. (1964). *Biochemistry*, **3**, 238.
- Kornienko, V. M. (1960). *Trans. Res. Inst. Biol., Kharkov State Univ.* no. 29, p. 29.
- Lang, N. & Sekeris, C. E. (1964). *Life Sci.* **3**, 161.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McQuorquodale, D. J., Veach, E. G. & Mueller, G. C. (1960). *Biochim. biophys. Acta*, **46**, 335.
- Mainwaring, W. I. P. (1968). *Biochem. J.* **110**, 79.
- Mansbridge, J. N. & Korner, A. (1963). *Biochem. J.* **89**, 15P.
- Matthaei, J. H., Jones, O. W., Martin, R. G. & Nirenberg, M. W. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 666.
- Maxwell, E. S. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1639.
- Medvedev, Z. A. (1966). *Protein Biosynthesis and Problems of Heredity, Development and Aging*, pp. 486 et seq. Edinburgh: Oliver and Boyd.
- Munro, A. J., Jackson, R. J. & Korner, A. (1964). *Biochem. J.* **92**, 289.
- Murthy, M. R. V. (1966). *Biochim. biophys. Acta*, **119**, 599.
- Nikitin, V. N. & Shereshevskaya, T. S. (1962). *Biokhimiya (Engl. Transl.)*, **26**, 918.
- Nirenberg, M. W. (1964). In *Methods in Enzymology*, vol. 6, p. 17. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Nirenberg, M. W. & Matthaei, J. H. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1588.
- Oeriu, S. (1964). *Advanc. Geront. Res.* **1**, 23.
- Oliver, I. T., Blumer, W. T. C. & Witham, I. J. (1963). *Comp. Biochem. Physiol.* **10**, 33.
- Rendi, R. & Hultin, T. (1960). *Exp. Cell Res.* **19**, 253.
- Sargent, J. R. & Campbell, P. N. (1965). *Biochem. J.* **96**, 134.
- Takayama, K., MacLennan, D. H., Tzagoloff, A. & Stoner, C. D. (1964). *Arch. Biochem. Biophys.* **114**, 223.
- Tissières, A. & Watson, J. D. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1061.
- Tsukada, K. & Lieberman, I. (1965). *Biochem. biophys. Res. Commun.* **19**, 702.