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THE INFLUENCE OF DIETARY PROTEIN ON THE INCORPORATION OF ^{14}C-GLYCINE AND ^{32}P INTO THE RIBONUCLEIC ACID OF RAT LIVER

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Two dietary factors influence the course of ribonucleic acid (RNA) metabolism in the liver. Protein intake plays a dominant role in determining the amount of RNA in the liver[1,2], whereas energy intake affects its rate of synthesis as measured by ^{32}P incorporation[2]. The influence which protein intake exerts over the amount of RNA in the liver does not appear to involve changes in the rate of RNA formation; thus the feeding of a protein-free diet causes a large reduction in the amount of RNA without affecting the absolute rate of ^{32}P uptake by RNA[2]. An alternative way in which the dietary supply of protein could influence the amount of RNA in the liver is by regulating its rate of breakdown. In the course of studying the influence of energy intake on ^{14}C-glycine incorporation into RNA, we obtained evidence strongly suggesting that the rate of RNA breakdown diminishes after a meal containing protein. This evidence emerged when we used rats which were trained to eat protein-containing or protein-free diets at fixed times each day; in consequence, it was possible to know with certainty whether the animals were in the post-absorptive state or were actively absorbing nutrients at the time of injection with isotopes. When the animals were fasting after meals containing protein, there was evidence of a considerable breakdown of RNA. After feeding protein, the picture rapidly changed to one which we have interpreted as indicating cessation of breakdown. These findings have led us to conclude that the intensity of protein synthesis determines the stability of liver RNA. This may have some bearing on the role of RNA in protein synthesis. A preliminary account of these experiments has already appeared[3].

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EXPERIMENTAL METHODS

Animals and diets. Young adult male albino rats were fasted overnight and those weighing 180–200 g were selected and housed individually under thermostatic conditions. The rats were fed on synthetic diets[4], either containing adequate amounts of protein or free from protein, for a period of 11 days before the isotopes were injected. The feeding routine was similar to that used previously[4]. During the first 7 days (preliminary period) both diets were fed at an energy level of 1200 cal./sq.m body surface area/day. Then for the remaining 4 days the carbohydrate content of each diet was varied to provide animals in different groups with either 800 cal. or 1600 cal./sq.m/day (low energy and high energy groups). In this way 4 dietary groups, two energy groups at each protein level, were obtained. Throughout the feeding period, the diets were given in two portions, the vitamins, minerals and most of the carbohydrate at 9 a.m., the rest (including any protein in the diet) at 5 p.m. The animals soon learned to consume these meals rapidly, so that on the morning after the last day of feeding they were all in the post-absorptive state. All of the rats on the protein-free diets and most of the rats on the protein-containing diets were then injected with ^{14}C-glycine and ^{32}P and remained fasting until killed. In addition, some of the rats which had been on the protein-containing diet were fed 2.5 g casein solubilized with 0.15 g $NaHCO_3$ one hour prior to injection with the isotopes. In this way rats actively absorbing amino acids from the gut could be compared with animals in the post-absorptive state.

Administration of isotopes and removal of liver. Each rat was injected at 9 a.m. with ^{32}P (10 μc inorganic phosphate/100 g body weight) and ^{14}C-2-glycine (10 μc/100 g body weight), one injection into each thigh. They were killed by exsanguination at 3, 6 or 9 h thereafter. The stomachs of the casein-fed rats were found to contain food at 3 and 6 h and often at 9 h after isotope injection, thereby ensuring a continuous supply of dietary amino acids throughout most of the labelling period. After perfusion with 0.9% saline, the liver was excised and homogenized in a Nelco blendor with about 5 vol. ice-cold 10% (w/v) trichloroacetic acid (TCA) for 3 minutes. The precipitate was separated by centrifuging, washed twice with chilled 10% TCA and then extracted with lipid solvents as described by MUNRO AND NAISMITH[4]. The dry lipid-extracted powder was used for quantitative determinations of protein N, ribonucleic acid (RNA) phosphorus, deoxyribonucleic acid (DNA) phosphorus, and for measuring the specific activities of ^{32}P in RNA, and of ^{14}C in protein and RNA. The TCA-soluble fraction of the liver was examined for specific activity of inorganic phosphorus and of glycine present in the free amino acid pool (free glycine). In some experiments in which the specific activity of ^{14}C in the acid-soluble adenine and guanine compounds was determined, perchloric acid (PCA) was used as a protein precipitant; 2 vol. *N* PCA were added to the liver and, after homogenizing, the residue was washed twice with 0.7 *N* PCA.

Fig. 1 summarizes the various estimations carried out on the liver and given in detail below.

Total protein N and RNAP. These were estimated by the methods described by MUNRO AND NAISMITH[4] and the amounts were expressed as mg/liver/100 g initial body weight.

Specific activity of inorganic phosphate and of RNAP. The activity of the inorganic phosphate was obtained by precipitation of $Mg(NH_4)PO_4$[5]. The specific activity of RNAP was obtained by the ionophoretic procedure of DAVIDSON AND SMELLIE[6], each ribonucleotide being eluted separately and its radioactivity and phosphorus content determined.

Radioactive carbon determinations. Glycine was isolated from liver protein, RNA adenine and guanine, acid-soluble adenine and guanine, and the free amino acid pool as the dinitrophenyl (DNP) derivative, using the procedure of CAMPBELL AND WORK[7] and of KROL[8], in which mixtures of DNP-amino acids can be separated on a Celite column with $CHCl_3$-butanol as the solvent, followed by purification on a Celite column developed with ether. The samples were counted on nickel planchettes under an end-window counter. The DNP-glycine was then dissolved off the planchette with $CHCl_3$-butanol and the DNP-glycine extracted with 1% (w/v)$NaHCO_3$ for colorimetric estimation. The specific activity of the glycine was expressed as counts/min/100 μg glycine. Self-absorption curves were obtained by plating out various thicknesses of a single sample of ^{14}C-labelled DNP-glycine. The purity of samples of DNP-glycine was periodically checked by paper chromatography, using the solvent of BLACKBURN and LOWTHER[9]; spots eluted from these chromatograms and counted gave specific activities similar to those of the specimens before paper chromatography. Since ^{32}P was also administered, the DNP-samples prepared from all sources were periodically checked for ^{32}P contamination by interposing a thin plate of copper foil between the sample and the end-window counter. Although the foil was permeable to radiation from ^{32}P, none was ever found in any of the DNP-samples.

Specific activity of free glycine in liver. About $^1/_3$ of the TCA-supernatant fluid from the liver was taken and the TCA removed by repeated ether extraction until the aqueous phase was pH 4–5. After evaporation to dryness, the residue was reacted with fluorodinitrobenzene and DNP-glycine isolated.

Specific activity of liver protein glycine. About 100 mg of the lipid-extracted TCA precipitate was hydrolyzed with 20 ml 6 *N* HCl for 14 h by refluxing on an oil bath. After removal of HCl, the

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product was reacted with fluorodinitrobenzene and DNP-glycine isolated. In some instances, the liver residue was first treated with 5% TCA at 100° for 30 min in order to remove nucleic acids (SCHNEIDER[10]), since these might be a possible source of glycine by hydrolysis of purine bases. Comparison of the radioactivity of glycine from samples so treated, with glycine from the untreated powder indicated a negligible difference in radioactivity if nucleic acids were not removed. Consequently, heating of the liver powder with TCA was not routinely carried out. This was further justified by subjecting a mixture of adenine and guanine to the action of 6 *N* HCl for 14 h at 110°. The yield of DNP-glycine, measured quantitatively by the procedure of KROL[8] represented breakdown of only 5.1% of the purine mixture to glycine.

Specific activity of purine glycine. Since the presence of glycine derived from protein would invalidate measurements of specific activity of ^{14}C in RNA samples, it was essential to ensure complete removal of protein before isolating the bases. This was carried out using the dodecyl sulphate procedure of KAY AND DOUNCE[11], in which the sample is first stirred with dodecyl sulphate in the presence of 0.9% NaCl. The protein can be precipitated by making the solution molar with respect to NaCl, and the crude nucleic acids are then precipitated from the supernatant fluid by addition of ethanol. The nucleic acid precipitate is taken up in water and again treated with dodecyl sulphate, followed by precipitation of protein. A third treatment with dodecyl sulphate is then carried out and the nucleic acids isolated. The last traces of protein are removed by dissolving the nucleic acids in 0.14% NaCl and adjusting the pH to 4.5, the precipitated proteins being removed by centrifugation. The yield of RNA by this procedure was rather poor, and, in accordance with the suggestion of DORNER AND KNIGHT[12], the dry, lipid-extracted powder was first extracted twice with the solution of dodecyl sulphate in *M* NaCl for 10 min at 100° before commencing to carry out the steps as recommended by KAY AND DOUNCE. This gave a higher yield.

The purified nucleic acids were incubated in 0.5 *N* KOH, the DNA precipitated by reducing the pH and the supernatant fluid adjusted to pH 3.5. From this the ribonucleotides were isolated by ionophoresis (DAVIDSON AND SMELLIE[6]). Since peptides have been described in filter-paper, the papers used for ionophoresis were first washed by the method of HANES, HIRD AND ISHERWOOD[13]. The effectiveness of this procedure was verified by submitting samples of the washed strips to ionophoresis, eluting the areas that would correspond to adenylic and guanylic acids, and after hydrolysis in 6 *N* HCl at 110° for 18 h, examining for amino acids by paper chromatography. After ionophoresis of the ribonucleotides, adenylic and guanylic acids were eluted separately and the bases separated from phosphorus using Dowex-50 resin columns[14]. The bases were then degraded to glycine at 200° in 11 *N* HCl for 18 h[15] and DNP-glycine prepared from the product. In

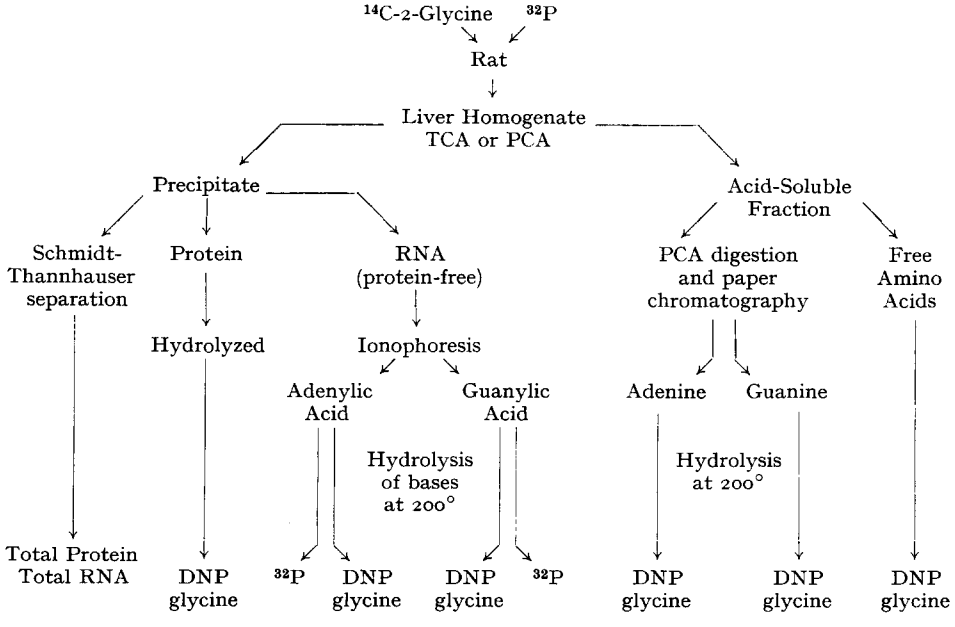

Fig. 1. Scheme of fractionation for the study of incorporation of ^{32}P and ^{14}C-2-glycine into the protein, RNA and acid-soluble compounds of rat liver.

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order to assure ourselves that the RNA preparation was free from protein, the eluates from the Dowex resin columns were hydrolyzed for 18 h in 6 *N* HCl at 110°. On paper chromatography of the product, only glycine, derived from partial degradation of the purines of RNA, was isolated. A test for peptides[16] was also carried out on the ionophoretic strips after development, with negative results.

Specific activity of glycine in acid-soluble purines. In order to separate these, the proteins and nucleic acids of the liver were precipitated with 2 vol. *N* perchloric acid (PCA) and the precipitate twice washed with 0.7 *N* PCA as rapidly as possible at 0°. The pH of the supernatant fluid was adjusted to 7 with KOH, and the precipitated perchlorate removed. The supernatant portion was then dried and digested with 72% PCA for 1 h at 100° to yield purine and pyrimidine bases. The PCA was neutralized with KOH and the precipitate removed. In order to separate the bases from brown degradation products of digestion, the product was extracted 8 times with 2 vol. butanol–water (86:14), which we have found to extract purine bases quantitatively. After separation, the butanol was evaporated to dryness and the residue taken up in water and applied to Whatman 3MM paper for 2-dimensional chromatography, the first solvent being the 5% Na_2HPO_4-*iso*amyl-alcohol (Light and Co., Technical grade) devised by CARTER[17]; the second solvent was the butanol: formic acid: water (77:10:13) system of MARKHAM AND SMITH[18]. The R_F values obtained agreed with these authors' findings, except that guanine moved in the first solvent with an R_F value of 0.45, whereas CARTER found a value of 0.02; this may be associated with impurities in the *iso*amyl-alcohol. The adenine spots were eluted in 0.1 *N* HCl at 37° for 18 h, and the guanine spots in 1.6 *N* HCl. After evaporation to dryness, the residues were hydrolyzed to glycine in 11 *N* HCl, as before, and the DNP-glycine prepared from the product.

Differential fractionation of liver. In some experiments the specific activity of the ribonucleotides was measured in different fractions of the liver cells 6 h after injection of ^{32}P. The methods of preparing the nuclei (citric acid procedure) and cytoplasmic fractions were similar to those used in a previous study[19].

Collection of urine and estimation of allantoin and creatinine. In some experiments, urine was collected for 24 h, using the method of separating urine from faeces described by THOMSON AND MUNRO[20]. The method of allantoin estimation was based on that of YOUNG AND CONWAY[21], as modified by FRIEDMAN, BYERS AND ABRAM[22]. In the modification of FRIEDMAN *et al.*, HCl hydrolysis of allantoic acid to glyoxylic acid and urea is carried out before formation of the phenylhydrazone by adding phenylhydrazine. It was found that the former step had to be prolonged to at least 5 min in the waterbath in order to obtain complete conversion. In the method of FRIEDMAN *et al.*, the addition of phenylhydrazine HCl and the final colour development with ferricyanide are carried out at —10° with addition of chilled reagents, but we found it more satisfactory to perform these at 20° in a water-bath and to use reagents at room temperature. Standards were run with each batch of estimations. A linear calibration curve was obtained, and it was confirmed that the colour fades on standing. Urinary creatinine was estimated by the method described by VARLEY[23].

RESULTS

The influence of dietary protein on the metabolism of liver RNA

The data on which we can base conclusions about liver protein and RNA metabolism in the present experiments consist of the total amount of protein and RNA per liver, the specific activity of the glycine in the protein, RNA purine bases and free amino acid pool of the liver, and the specific activity of ^{32}P in the ribonucleotides and the inorganic phosphate of the liver (Fig. 1). From these we can compute the specific activity of ^{14}C-glycine in the liver protein and the purine bases as a percentage of the specific activity of glycine in the free amino acid pool of the liver; similarly, the specific activity of ^{32}P in the ribonucleotides can be related to that of inorganic phosphate. These relative specific activities allow for differences in the activities of the precursor pools of different animals, and permit us to compare the proportion of atoms renewed in the liver protein or RNA in the different nutritional groups. In order to complete the picture, it is necessary to allow for the total amount of protein and RNA in which incorporation is taking place; thus an animal with a high relative specific activity and a small amount of RNA may not be synthesizing a greater number of new mole-

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cules than an animal with a low relative specific activity but with a larger amount of RNA in its liver. As a measure of the absolute rate of replacement per liver, the relative specific activity has therefore been multiplied by the total amount of protein N or RNA P in the liver expressed as mg/100 g initial body weight. This is referred to as "total relative activity".[24].

At the time of injection with the isotopes, the rats were nutritionally in one of three conditions. One group had been fasting overnight after a few days feeding on a protein-free diet, and a second group were fasting after a diet containing protein. In addition, some animals on the protein-containing diet were fed more casein just prior to injection. Thus we have two groups in the post-absorptive state after diets of different protein content, and one group actively absorbing amino acids from dietary protein. Animals from each group were killed at 3, 6 and 9 h after injection. The whole experiment was replicated three times using rats fed on diets of low energy content and three times with rats on high energy diets (see experimental section). Consistent effects of protein intake on RNA metabolism were obtained in all six replications, irrespective of whether the animals had been fed at the low or the high level of energy intake. The results obtained at each energy level are therefore combined in Fig. 2.

Glycine incorporation into liver protein under these different nutritional conditions will be considered more fully elsewhere[25]. At present, it is sufficient to note (Fig. 2) that the total amount of glycine incorporated into liver protein was of similar magnitude in the two fasted groups, irrespective of the protein content of the preceding diet. However, the rats fed protein at the time of isotope injection exhibited much enhanced uptake of glycine. This agrees with nutritional evidence that utilization of dietary protein is confined to a short period after its ingestion, since omission of an essential amino acid from the diet cannot be compensated for by feeding the missing amino acid a few hours later[26].

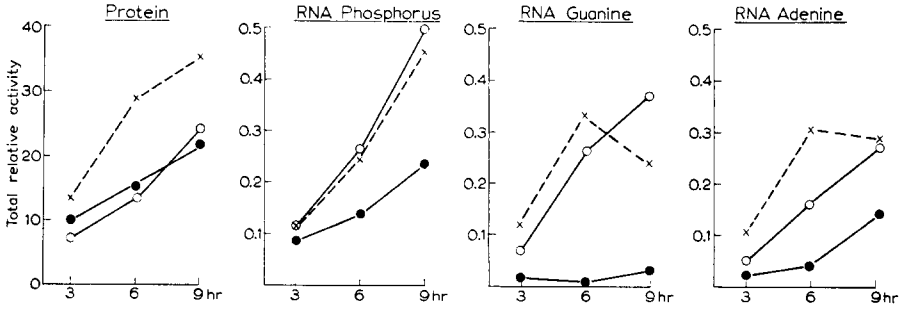

Fig. 2. Incorporation of ^{14}C-2-glycine into liver protein and of ^{32}P and ^{14}C-2-glycine into RNA under various nutritional circumstances. Uptake is expressed in units of total relative activity (see text for method of calculation). The data are the means of six replications of the experiment. O—O Fasting after protein-free diet. ●—● Fasting after protein-containing diet. ×--× Fed protein after protein-containing diet.

Incorporation of labelled precursors into RNA was affected by protein intake in quite a different manner (Fig. 2). The animals fasting after the protein-containing diet showed about half the uptake of ^{32}P obtained with the rats fasting after the protein-free diet. When protein was fed to the animals at the time of isotope injection, uptake of ^{32}P into RNA rose to about the level found in the groups receiving the

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protein-free diet. Incorporation of ^{14}C-glycine into RNA shows the same picture, except that the changes are more striking. The group fasted after the protein-containing diet had an uptake of ^{14}C into adenine which was about one-third of that exhibited by the group on the protein-free diet, and uptake of ^{14}C by guanine was even lower, averaging about one-tenth of the uptake on the protein-free diet. On re-feeding protein, incorporation into guanine rose to about the level obtained with the protein-free diet and incorporation into adenine to a somewhat higher level. It should be emphasized that these dramatic changes in glycine uptake are not artefacts due to the method of calculating total relative activity, but can be seen in the specific activities of the purine bases without reference to the activity of the precursor pool of glycine. For example, at 6 h after injection of labelled glycine, the specific activities of the glycine isolated from adenine and from guanine were 100 and 156 respectively for the group fasted after the protein-free diet, 15 and 5 for the group fasted after the protein-containing diet, and 63 and 62 for the group fed protein just prior to injection of the isotope.

From these data it is apparent that rats which had been receiving no dietary protein for several days incorporated as much ^{32}P and, in the case of guanine, as much ^{14}C-glycine into liver RNA as did rats which had been on the protein-containing diet and were eating protein at the time of injection with the isotopes. This lack of association between dietary protein supply and RNA metabolism agrees with the results of previous experiments[2] carried out on rats *receiving food during the period of labelling*; in these earlier studies the rate of ^{32}P uptake by liver RNA was found to be independent of the amount of protein in the diet. In contrast to this, the protein content of the diet has a profound effect on incorporation of both ^{32}P and ^{14}C-glycine when the rats are *in the post-absorptive state throughout the period of labelling*; thus uptake of isotopes by animals fasting after the protein-containing diet was much inferior to

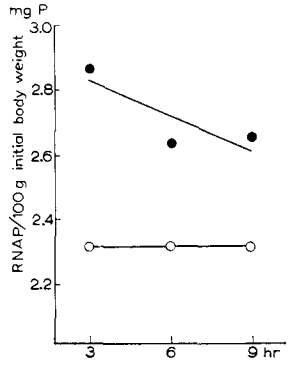

Fig. 3.

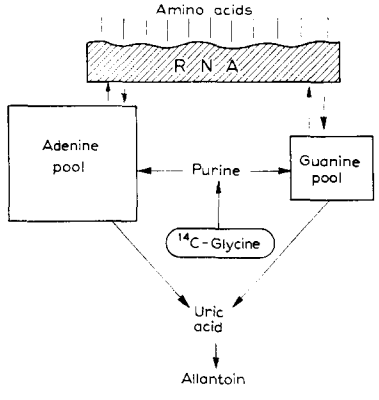

Fig. 4.

Fig. 3. Changes in the amount of RNA per liver during fasting after a protein-containing diet (upper line) and after a protein-free diet (lower line). The data are the means of three experiments carried out with diets low in energy content; similar results were obtained with diets of higher energy content. The combined data at low and high energy levels indicate that the protein content of the preceding diet has a statistically significant effect on the loss of RNA from the liver during fasting. O—O Fasting after protein-free diet. ●—● Fasting after protein-containing diet.

Fig. 4. Scheme illustrating the effect of RNA breakdown on the pools of precursor compounds.

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uptake by the group fasting after the protein-free diet (Fig. 2). It is possible to explain these effects of protein intake on incorporation of isotopes by considering the way in which the amount of RNA in the liver is affected by fasting after each type of diet. Fig. 3 shows that the rats fed on the protein-containing diet started the fast with more RNA per liver than did the rats fed on the protein-free diet, and moreover there was a significant fall during the 9-h period elapsing after injection of the isotopes. On the other hand, the rats on the protein-free diet started fasting with less RNA per liver but the amount was not further reduced by fasting for the 9-h period. This picture has been confirmed in a separate experiment in which the period of fasting was extended to 24 h (Table I); it is also in agreement with the findings of KOSTERLITZ[27] for changes in the total nucleic acid content of the liver during fasting after diets of differing protein content. From this evidence we may conclude that fasting after a protein-containing diet results in an accelerated breakdown of RNA, and thus the most likely explanation for the low uptake of isotopes by RNA in such animals is that the labelled RNA precursors become diluted by the breakdown products entering the same pool (Fig. 4). When rats from the same dietary group were fed protein just prior to injection with the isotopes, uptake of ^{32}P and ^{14}C-glycine rose rapidly to the levels obtained with rats on the protein-free diet, and in such animals liver RNA did not fall significantly (Table I). This suggests that, as soon as amino acids start to be absorbed from the gut, RNA breakdown is halted with consequent cessation of dilution of the labelled RNA precursors. In other words, protein intake influences the amount of RNA in the liver by regulating its rate of breakdown.

TABLE I

CHANGES IN THE AMOUNT OF RNA IN THE LIVERS OF RATS FASTED OR FED PROTEIN DURING A 24-H PERIOD FOLLOWING DIFFERENT DIETS

(The data are the means of three replications of the experiment. Statistical analysis shows that only the group fasted after the protein-containing diet lost a significant amount of RNA.)

Preceding diet		*Fed during period of observation*	*mg RNA P/100 g initial body weight*		
Energy level	*Protein content*		*0 h*	*24 h*	*Difference*
High	Protein-free	Nil	2.33	2.63	+0.30
	Protein-containing	Nil	3.06	2.52	—0.54
	Protein-containing	Protein	3.06	3.01	—0.05
Low	Protein-free	Nil	2.37	2.10	—0.27
	Protein-containing	Nil	2.62	1.78	—0.84
	Protein-containing	Protein	2.62	2.52	—0.10

In support of this interpretation of the data, we have examined the effects of diet on labelling in the precursor pools, on the uptake of ^{32}P by the RNA of different fractions of the liver cell and on allantoin excretion.

Uptake of ^{14}C-glycine by RNA precursors

If the low uptake of isotopes by RNA in animals fasted after a protein-containing diet is in fact due to dilution with breakdown products, one would expect to find evidence

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of such dilution effects among the precursors of RNA. As a first approximation to the necessary data, we examined glycine uptake by the pooled adenine and guanine compounds of the perchloric acid soluble fraction of liver. It has been shown by BENNETT AND KRUECKEL[28] that most of the acid-soluble adenine is present in the form of adenosine mono-, di- and triphosphates, and that guanine takes corresponding forms almost exclusively. After administration of labelled adenine, they observed that the pools of adenosine compounds rapidly reached equilibrium, and similarly for the guanosine derivatives. Examination of the total adenine and total guanine of the perchloric-acid soluble fraction of liver will therefore provide a reasonable approximation to the general state of the acid-soluble adenine and guanine precursors of RNA, if we accept adenosine and guanosine phosphates as likely precursors.

Since the action of dietary protein on RNA metabolism was not found to differ appreciably at low and high caloric intakes, the study of changes in labelling in the precursor pools with variations in protein intake was made at an intermediate caloric intake, namely 1200 cal/sq.m/day. The total amounts of adenine and guanine in the acid-soluble fraction were not measured; consequently the results are expressed, not as total relative activities, but as the specific activity of glycine isolated by hydrolysis from these purines relative to the specific activity of free glycine. In the case of rats fasted after the protein-containing diet, the uptake of glycine by the adenine and guanine of the acid-soluble pool was much less than in the case of rats fasted after the protein-free diet (Fig. 5). On feeding protein just prior to injection, incorporation of glycine was markedly stimulated. The changes observed in RNA labelling are thus, in the main at least, reflections of corresponding changes in the precursor pools of adenine and guanine compounds.

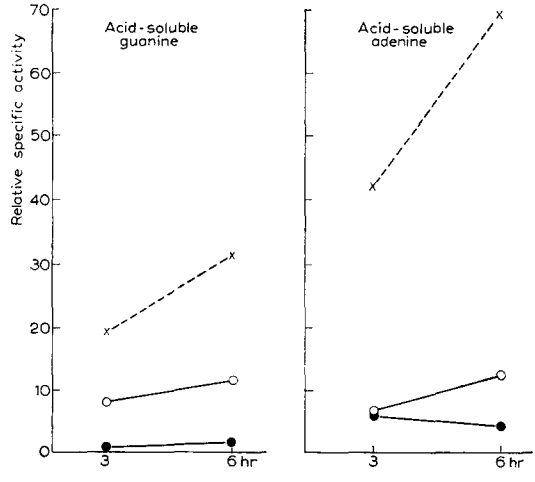

Fig. 5. The effect of diet on the uptake of ^{14}C-2-glycine by the total guanine and adenine of the acid-soluble fraction of rat liver. The data are the specific activities of purine glycine as percentages of the specific activity of free glycine at each time interval. O——O Fasting after protein-free diet. ●——● Fasting after protein-containing diet. ×----× Fed protein after protein-containing diet.

The hypothesis of variations in RNA breakdown receives further support when we consider the relative effects which dilution with RNA breakdown products would have on the acid-soluble adenine and guanine compounds. The pool of adenine compounds is much larger than that of guanine compounds (*cf.* Fig. 4), whereas liver RNA contains slightly more guanine than adenine[29]. Breakdown of RNA would therefore have a greater diluent effect on the pool of guanine compounds. Examination of the relative uptakes of radioactive glycine into guanine and into adenine of the acid-

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TABLE II

THE EFFECT OF DIFFERENT NUTRITIONAL CONDITIONS ON THE RELATIVE UPTAKE OF ^{14}C-GLYCINE BY THE PURINE BASES AND OF ^{32}P BY THE PURINE RIBONUCLEOTIDES OF RAT LIVER

Statistical analysis shows that the guanine/adenine ratio for ^{14}C uptake was significantly affected by diet in the case of the acid-soluble purines and the purines of RNA; diet had no significant effect on the pattern of ^{32}P uptake. (The data for the acid-soluble purines are taken from the experiment recorded in Fig. 5; the data for ^{14}C-glycine and ^{32}P uptake by RNA are from the experiments described in Fig. 2. The ratios obtained at different times after isotope injection followed essentially the same pattern and have been averaged.)

Previous diet	*Fed after isotope injected*	*Uptake of ^{14}C-glycine*		*Uptake of ^{32}P by RNA*
		Acid-soluble guanine/adenine ratio	*RNA guanine/adenine ratio*	*Guanylic/adenylic acid ratio*
Protein-free	Nil	1.15	1.41	0.94
Protein-containing	Nil	0.26	0.48	0.96
Protein-containing	Protein	0.47	0.96	0.92

soluble fraction and of RNA (Table II) shows that this prediction has been fulfilled in the present experiments. Fasting after the protein-containing diet reduced the uptake of glycine into guanine much more severely than into adenine, as evidenced by a fall in the guanine/adenine ratio. The ratio rose again when protein was fed, *i.e.*, when dilution from RNA breakdown products was held in check. It is of interest to note that uptake of ^{32}P was of the same order of magnitude in all four nucleotides of RNA, irrespective of changes in the total amount of ^{32}P incorporated in response to different nutritional conditions. Table II shows the absence of a dietary effect on the guanylic/adenylic acid ratio, which is representative of the picture obtained with ^{32}P incorporation into the other nucleotides.

Isotope uptake by different liver cell fractions

Rats fasting after a protein-containing diet exhibit a low uptake of isotopes into liver RNA (Fig. 2). If this is due to dilution of RNA precursors by RNA breakdown products, it is likely that precursors of RNA in all parts of the liver cell will be affected. For this reason, we examined incorporation of ^{32}P into the RNA of different cell fractions under various dietary conditions (Table III). In the case of rats fasting after the protein-containing diet, the RNA in all cell fractions exhibited a low uptake of the

TABLE III

INCORPORATION OF ^{32}P INTO THE RIBONUCLEIC ACID OF DIFFERENT FRACTIONS OF THE LIVERS OF RATS RECEIVING VARIOUS DIETS AND KILLED 6 H AFTER INJECTION OF 50 μC OF ^{32}P

The data are the mean specific activities of the ribonucleotides in each fraction relative to the specific activity of liver inorganic phosphate; each dietary group consisted of 3 rats.

Previous diet	*Fed after isotope injected*	*Nuclei*	*Mitochondria*	*Microsomes*	*Cell sap*
Protein-free	Nil	33.0	4.7	6.7	9.4
Protein-containing	Nil	18.3	2.7	3.0	5.9
Protein-containing	Protein	38.2	8.5	9.4	12.4

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isotope. On feeding protein to such animals, ^{32}P incorporation rose proportionately in the different fractions. This may be contrasted with the effect of changes in protein intake on the total amount of RNA in these fractions. In previous studies[19], it was found that only the RNA of the microsomal fraction (sedimented at 20,000 *g*) and possibly the nuclear RNA underwent alterations in amount when protein intake was altered. This finding is not compatible with the isotopic data given in Table III if we believe that the increased ^{32}P uptake observed after feeding protein is due to an increased rate of RNA synthesis; in that case, increased ^{32}P incorporation ought to be confined to the fraction changing in amount. The quantitative data and the isotopic evidence can, however, be reconciled if we assume that variations in dilution by microsomal RNA breakdown affect the labelling of a precursor pool common to all cell fractions.

Diet and allantoin excretion

Since allantoin is the principal excretory product of purine metabolism in the rat, it was thought that urinary output of allantoin might reflect changes in the rate of RNA breakdown. Urine was collected from rats over a 24 h period of fasting following diets containing protein or free from protein; in addition, one group was fed protein throughout the 24-h period. In order to minimize variations in allantoin output resulting from incomplete voiding of urine, the results were expressed in relation to creatinine output, which was assumed to be unaffected by diet. Table IV shows that the feeding of protein reduced output significantly in comparison with the output of rats fasting after the protein-containing diet. This is compatible with the hypothesis that RNA breakdown is diminished by feeding protein. In contrast to the findings in these two groups, the allantoin excretion of rats fasting after the protein-free diet was found to vary significantly with the energy content of the preceding diet. Energy level in the preceding diet did not have this effect on rate of loss of RNA from the livers of fasting animals (Table I), and the effect of previous energy intake on allantoin excretion is presumably due to other causes.

TABLE IV

THE URINARY OUTPUT OF ALLANTOIN BY RATS FASTED OR FED PROTEIN DURING A 24-H PERIOD FOLLOWING DIFFERENT DIETS

(The data are the means of six replications of the experiment. Statistical analysis shows that the feeding of protein during the period of collection caused a significant reduction in allantoin excretion and that energy intake had a significant effect on output after the protein-free diet).

Previous diet	*Fed during period of collection*	*mg Allantoin/mg Creatinine*	
		High energy group	*Low energy group*
Protein-free	Nil	3.97	5.93
Protein-containing	Nil	4.40	4.64
Protein-containing	Protein	4.02	3.99

DISCUSSION

The participation of RNA in protein synthesis was originally suspected because of the large amount of RNA found in cells with a high rate of protein synthesis. Nevertheless,

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attempts to demonstrate a relationship between rate of protein synthesis and uptake of isotopes by RNA have sometimes yielded negative results. For example, HOKIN AND HOKIN[30] studied ^{32}P uptake by the RNA of pigeon pancreas and found that changes in the rate of amylase synthesis were not necessarily accompanied by changes in isotope incorporation. Similarly, DE DEKEN-GRENSON[31] was unable to relate RNA synthesis to protein synthesis in the pancreas and oviduct.

The present experiments indicate the occurrence of a similar dissociation between protein synthesis and RNA synthesis in the case of the liver. Comparison of rats receiving a meal of protein at the time of isotope injection with animals fasting after several days on a protein-free diet revealed a considerable stimulation of ^{14}C-glycine incorporation into the liver proteins of the protein-fed group (Fig. 2). Nevertheless, the two groups showed an essentially similar uptake of ^{32}P and of ^{14}C-glycine into RNA. This finding agrees with the results of previous studies[2] in which the animals were fed on protein-free or protein-containing diets during the period of labelling. Under these conditions, the absolute rate of ^{32}P uptake was found to depend on the energy content of the diet but not on its protein content. However, our present experiments also show that rats *fasting* after a diet containing protein had a much reduced uptake of isotopes into RNA (Fig. 2). Unlike the other two groups, the RNA content of the livers of these animals was diminishing rapidly during the period of labelling (Fig. 3 and Table I), and it has been concluded that the low isotope uptake of this group was due to dilution of precursors with breakdown products. Subsequent experiments showed that the purine bases of the precursor pool did in fact exhibit a low uptake of glycine under these conditions (Fig. 5), that reduction in the labelling of adenine and guanine differed in the way one would anticipate from dilution (Table II), that changes in ^{32}P uptake were spread over all subcellular fractions in a manner compatible with dilution of a common precursor pool (Table III) and that allantoin excretion was greater in fasting than in protein-fed animals (Table IV). Since evidence of dilution was swiftly obliterated on feeding protein, it has been concluded that breakdown of RNA is partly or wholly suspended while the dietary amino acids are being absorbed from the gut.

Presumably there is some sort of relationship between the stability of liver RNA and the availability of amino acids for protein synthesis, so that a given level of amino acid supply confers stability on a certain quantity of RNA. Thus in the case of protein-depleted rats, the diminished amount of RNA in their livers is equilibrated with the reduced supply of amino acids in circulation. Similarly, the larger amount of RNA in the livers of rats receiving a protein-containing diet can be maintained in a stable state so long as the animals continue to receive protein. It is during transition from one nutritional state to another (*e.g.* during a short period of fasting after a diet containing protein) that we observe effects due to imbalance between amino acid supply and the amount of RNA. These effects will continue to be evident until a new equilibrium between the amount of RNA and the supply of amino acids is achieved. This is brought out quite strikingly in one of our previous experiments[2] in which rats were transferred from a normal diet to a protein-free diet. During the first day of protein deficiency, the RNA content of the liver fell sharply and thereafter became plateaued. Uptake of ^{32}P by RNA was low during the first day of the protein-free diet (dilution effect), but rose to a high level as soon as the RNA content of the liver ceased to fall.

The picture of RNA metabolism which emerges from the present and previous studies[2] is thus one of steady synthesis of RNA in proportion to the supply of energy,

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variations in the amount of RNA (in the microsomes[19]) being brought about by alterations in the rate of its breakdown in response to changes in the supply of amino acids available for protein synthesis. This view of the relationship between RNA metabolism and protein synthesis would account for the fact that tissues with a high rate of protein synthesis have a large content of RNA, yet do not exhibit a raised rate of RNA formation[32].

In view of the recent discovery[33] of an enzyme system which can polymerize nucleoside diphosphates to RNA-like compounds, it is of interest to consider whether our present data are compatible with RNA precursors of this type. Table II shows that the various dietary conditions used in our experiments affected the labelling of adenine and of guanine differently, whereas the specific activity of ^{32}P was essentially similar in all four ribonucleotides under these different nutritional conditions. This is precisely what one would expect if ADP and GDP are immediate precursors of RNA. The recent studies of BRUMM, POTTER AND SIEKEVITZ[34] show that the acid-labile atoms of ADP and GDP, as well as those of the other nucleosides diphosphates, are in facile equilibrium. In consequence, even if RNA breakdown leads to unequal dilution of the pools of ADP and GDP, the terminal P atoms will at once equilibrate and result in similar specific activities of ^{32}P. However, the rate of conversion of adenine to guanine is much slower[28], so that unequal dilution of the bases of ADP and GDP during RNA breakdown will persist. It may be noted that the P atoms of AMP and GMP are not so readily exchanged as are the high energy P atoms[34]; consequently they are less acceptable as likely precursors under our experimental conditions. OCHOA[33] has shown that his enzyme system is reversible, and can lead to nucleoside diphosphate formation from the RNA polymer. It is thus possible that the breakdown products responsible for the dilution of RNA precursors in our experiments are in fact ADP and GDP themselves. If so, we have the interesting situation in which the point of equilibrium between the nucleoside diphosphates and RNA is determined by the supply of amino acids available for protein synthesis.

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SUMMARY

1. Rats were maintained on diets either containing adequate amounts of protein or free from protein and, after fasting overnight, were injected with ^{32}P and with ^{14}C-2-glycine. They were killed at 3, 6 and 9 h thereafter. A further group of rats which had received the protein-containing diet were fed protein at the time of injection with the isotopes.

2. The uptake of glycine into liver protein was essentially similar in the two groups in the post-absorptive state, but rose considerably when protein was fed at the time of injection.

3. Labelling of RNA with ^{32}P and with glycine was much reduced in rats fasted overnight after the protein-containing diet. On feeding protein to this group, uptake of both isotopes rose to the level found with the protein-free diet.

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4. It is suggested that the low level of labelling in the groups fasted after the protein-containing diet was due to breakdown of RNA as soon as the supply of amino acids from the gut ceased, the products of breakdown causing dilution of isotopically labelled precursors of RNA in the acid-soluble fraction of liver. The feeding of protein, by restraining RNA breakdown and thus terminating dilution of these precursors, restored the level of labelling in RNA. This hypothesis is supported by a study of the effect of diet (a) on changes in the RNA content of the liver, (b) on ^{14}C-uptake by the purines of the acid-soluble fraction of liver, (c) on the uptake of ^{32}P by the RNA of different liver cell fractions and (d) on allantoin excretion.

5. It is concluded that the rate of synthesis of liver RNA is a function of the available energy, whereas the stability and therefore amount of RNA in the liver is determined by the supply of amino acids for protein synthesis.

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