STUDIES ON PROTEIN SYNTHESIS IN VITRO

VI. Incorporation and Release of Amino Acids in Particulate Preparations from Livers of Rats*

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(Received for publication, March 22, 1957)

ABSTRACT

The release of radioactive amino acid from the particulate fractions separated from the prelabelled livers of rats by centrifugation has been studied under various conditions. Although pure fractions may not have been obtained, great differences in behavior were observed. In the mitochondria and nuclei dinitrophenol (10^{-4} M) causes an inhibition of release, but in microsomes the opposite effect is observed. When the incubation medium is fortified with ATP and phosphocreatine, release is inhibited. In microsomes and nuclei the inhibition proceeds to the extent that the incorporation of preformed radioactive amino acid occurs. Protein is synthesized at a rapid rate. In incubations longer than 1 hour there is always a release of radioactive amino acid. It is concluded from these results that the interpretation of release data from slices or systems such as those studied is impossible without further information concerning some of the unknown variables. The most important unknown is the specific activity of the "free" amino acid in the particulates and the effect of carrier amino acid in the medium of this specific activity.

INTRODUCTION

Simpson (1) showed that the release of radioactivity from the labelled proteins of rat liver slices was depressed under anaerobic conditions as well as by cyanide and 2,4-dinitrophenol (DNP). The implication from these experiments, which have been confirmed by Steinberg and Vaughan (2), is that the breakdown of tissue proteins is not due simply to the hydrolytic splitting of their peptide bonds catalyzed by tissue cathepsins; but it is linked, in some manner direct or indirect, with protein synthesis and the supply of energy. Steinberg and Vaughan (2) extended the original work by showing that β-thienylalanine and *o*-fluorophenylalanine inhibited the release of phenylalanine.

* This work was aided by grants from the American Cancer Society, and the School of Medicine, University of California.

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Moreover, the latter inhibitor also inhibited the release of alanine from the slice protein. It was also demonstrated by these workers that along with the inhibition of release, there was also a more or less proportional inhibition of formation of non-protein nitrogen, whether the release was inhibited by DNP, by analog, or by anaerobic conditions.

We have been able to confirm the work of Simpson (1), prelabelling the livers by feeding rats either yeast labelled with S^{35} or *Rhodospirillum rubrum* labelled with C^{14} (3), and, in an attempt to gain further insight into this phenomenon, we have investigated the release of labelled amino acids from the nuclear mitochondrial, and microsomal fractions of livers of rats in order to see if these fractions behave differently from one another in releasing amino acids from their proteins, as they do with regard to the incorporation of amino acids into their proteins (4–6).

From the present results it is apparent that the release of radioactive amino acids from nuclei, mitochondria, and microsomes is different for each particulate fraction and also with the different conditions of incubation. When conditions which stimulate protein synthesis exist during the incubation of microsomes, there may actually be incorporation rather than release of amino acid. It therefore becomes necessary to reconsider the interpretation of the results of Simpson and of Steinberg and Vaughan.

Methods

Rhodospirillum rubrum grown on a medium containing C^{14}-bicarbonate (3) was given by stomach tube to male black and white hooded rats from 3 to 4 months of age. Each rat received about 10^8 counts/min. Forty-eight or 72 hours later, the livers of the rats were subjected to perfusion to remove most of the blood. The organ was then dissected out as quickly as possible, blotted, weighed, cut into small pieces, and transferred into five times its weight of fresh, ice-cold homogenizing medium (H) in a Dounce-type homogenizer (7). This consisted of 8.5 per cent sucrose containing 0.0025 M $MgCl_2$, 0.001 M $MnCl_2$, and 1.0 mg./ml. of mixed amino acids at pH 7.3. The composition of the mixture is given as a footnote to Table III. The homogenate was filtered through silk or gauze to remove fibre and the nuclear, mitochondrial, and microsomal fractions were separated by differential centrifugation after the technique of Schneider and Hogeboom (8). The pellets so obtained were washed, each in its incubation medium containing excess carrier amino acid, to give a low initial level of radioactivity in the medium. It cannot be claimed that these fractions are completely pure since no doubt there is some admixture of the components.

The nuclei were suspended by gentle homogenization in the incubation medium (I) described in the footnote to Fig. 1, containing 0.002 M $CaCl_2$ and 0.01 M sodium citrate. The microsomes and mitochondria were suspended in a medium lacking the calcium and citrate, but containing 0.001 M $MnCl_2$ and 0.0025 M $MgCl_2$. All media were gassed with either 95 per cent O_2, 5 per cent CO_2 or 95 per cent N_2, 5 per cent CO_2, and were at pH 7.2, except in the experiment reported in Table II. They also contained carrier amino acids like medium H, except in the experiments reported in Table III. In those

cases in which yeast-S^{35} was used to do the initial labelling, sulfur amino acids only were used as carrier as indicated below Fig. 1.

The nuclei were usually ready for incubation within 45 minutes of the death of the rat. The mitochondria took about 75 minutes to prepare and the microsomes about 180 minutes. In most experiments the nuclei and mitochondria were kept at 0°C. until the microsomes were ready, but occasionally incubations were started immediately.

In some experiments, as noted in the tables and figures, adenosinetriphosphate (ATP), phosphocreatine (PC), 2,4-dinitrophenol (DNP), or the soluble enzyme prepared as described by Hoagland, Keller, and Zamecnik (9) was added to the media. All addenda were at pH 7.2, and the necessary small volumes of water were added to the controls.

Triplicate samples of the particulate fractions were removed at various time intervals during the incubation, and the reaction stopped by boiling with 0.25 M acetate buffer at pH 4.7. The protein was spun down and the supernatant poured off. The protein was washed once with 1 ml. of a solution of the mixed amino acids and twice with water. The volume of the combined washings and supernatant was made up to 8 ml. with water, mixed, and triplicate 2 ml. samples were pipetted into plastic planchets, dried, and counted in a gas-flow Geiger-Müller counter with an end window. The residual protein was washed twice with 10 per cent trichloroacetic acid (TCA), once with hot TCA, once with hot alcohol, and once with hot alcohol-ether, dried with ether, sedimented on to aluminum planchets, and counted. The specific activity was calculated after corrections had been made for background counts and for self-adsorption.

Similar experiments were performed after administration to the rats of yeast grown on a medium containing S^{35}-sulfate. In some of these experiments the S-amino acid content of the medium was determined by counting the sulfur from these amino acids. A known amount of carrier sodium sulfate was added and the inorganic sulfate was precipitated by the addition of the stoichiometric amount of barium chloride solution. The sulfur of the amino acids was then converted to inorganic sulfate with Pirie's reagent, and the specific activity obtained by counting and titrating the benzidine salt (10).

The protein content of the samples was determined by nesslerization after micro-Kjeldahl digestion of the TCA precipitate. The results are given either as total counts released, or as the percentage of the counts released from the protein.

It will be noted (see footnotes to Figs. 1, 2, and 6) that even at the beginning of incubation most samples contained a significant amount of non-protein radioactive material. In the supernatant fraction from the livers this activity was so high, relative to the activity found in the protein, that it proved impossible to carry out satisfactory experiments with this fraction. In computing the results this zero time non-protein activity has been treated as a blank value, being subtracted from all subsequent samples.

RESULTS

The results given in Table I indicate that in an incubation period of 4 hours the nuclear, mitochondrial, and microsomal fractions each released radio-

activity (C^{14}) into the medium, whether O_2/CO_2 or N_2/CO_2 was present as gas phase. This release is an enzymatic process, for very little amino acid was released when the incubation was carried out in melting ice. Thus in a 4 or 6 hour incubation under O_2/CO_2 at 0° there was a rise in non-protein counts in the medium of less than 3 per cent of the initial value no matter which of the three particulate fractions was employed.

The percentage of counts in the protein which was released varied considerably with each preparation of the fractions. Thus the percentage decrease in release resulting from incubation under nitrogen varied in five experiments with the nuclei from 19 to 47 per cent, with the mitochondria from 9 to 37 per cent, and with the microsomes from −28 to −45 per cent. Some of the varia-

TABLE I

*Effect of Anaerobiosis on the Release of Radioactivity from the Proteins of Particulates from Livers of Rats**

	Radioactivity released, *per cent*‡		
	Gas phase		Decrease in release in N_2
	O_2/CO_2	N_2/CO_2	
Nuclei	8.7	6.5	26
Mitochondria	7.8	5.3	31
Microsomes	3.7	5.2	−41

* The livers were previously labelled by feeding the animals *Rhodospirillum rubrum* labeled with C^{14}. Fractionation was carried out by centrifugation from sucrose as indicated in the text. The conditions of incubation are given below Fig. 1.

‡ The activity released refers to the per cent of the radioactivity transferred from the protein to the non-protein fraction after 4 hours' incubation at 37°C. The range of values obtained in five experiments is given in the text.

bility may be due to cross-contamination among the fractions, but this is insufficient to mask the differences observed. Perhaps the pure fractions would have shown even greater differences among themselves. Quite clearly incubation under nitrogen, as compared with oxygen, caused an inhibition of release from the nuclei and mitochondria, but a stimulation of release from the microsomes. Similarly in experiments in which the prelabelling was carried out with yeast-S^{35}, DNP caused an inhibition of release of amino acid from the nuclei, but stimulation in release from the microsomes (Fig. 1). The same phenomena are shown by the results of experiments in which labelling was carried out with C^{14} (Fig. 2). In addition, it is demonstrated that DNP inhibits the release from mitochondria as well as from the nuclei.

Thus the experiments with S^{35} labelling, in which the substance initially released is necessarily amino acid, conform to those with C^{14}, in which it is

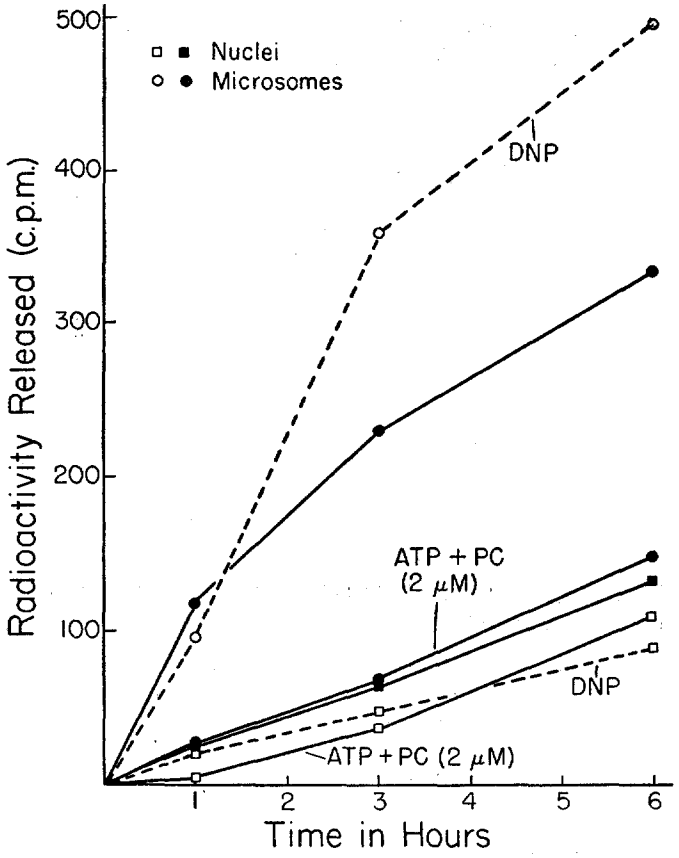

FIG. 1. Effect of conditions on the "release" of label from nuclei and microsomes isolated by centrifugation from livers of rats previously labelled by feeding yeast-S^{35}.

At zero time the states of systems were as follows: (*a*) nuclei, 15 mg. of protein with a specific activity of 225 C.P.M. per mg. and 220 C.P.M. in non-protein fraction; (*b*) microsomes, 5 mg. of protein with a specific activity of 3100 C.P.M. per mg. and 505 C.P.M. in non-protein fraction. All samples were incubated for the times indicated under 95 per cent O_2; 5 per cent CO_2 in (for nuclei) a medium containing 0.04 M sodium bicarbonate at pH 7.2, 0.002 M $CaCl_2$, 0.10 M KCl, 0.04 M NaCl, and 1.0 mg. per ml. of mixed sulfur amino acids (0.6 mg. per ml. of L-methionine, 0.4 mg. per ml. of L-cystine, plus cystine to saturation). For the microsomes the $CaCl_2$ was replaced with 0.001 M $MnCl_2$ and 0.0025 M $MgCl_2$. When used the DNP was 10^{-4} M, and ATP and PC, 1 or 2 μM per sample. Activity released refers to S^{35} in non-protein fraction.

possible that some of the label in the particulates is initially present as fat and carbohydrate and may be released as such. In the following discussion we shall assume, therefore, that the major part of the radioactivity released or taken up by the particulates was in the form of free or "activated" amino acid.

It is clear that the results with the particulates make a simple interpretation of the phenomenon of release impossible.

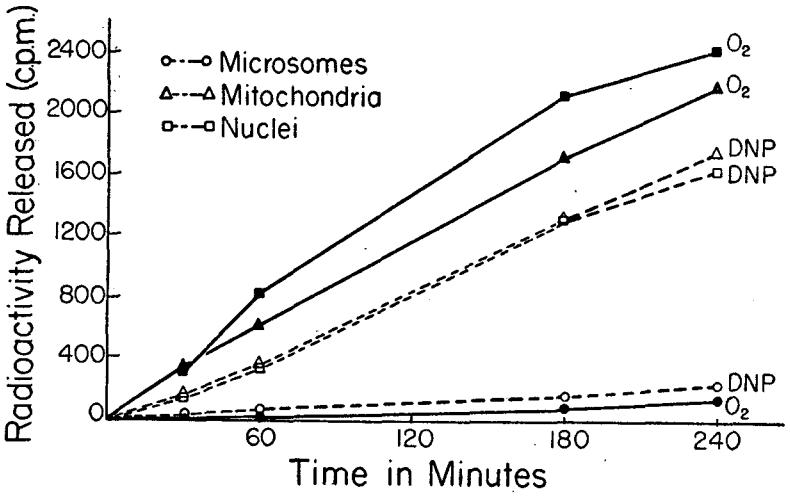

FIG. 2. Effect of DNP on "release" of label from fractions isolated by centrifugation from livers of rats previously labelled by feeding *Rhodospirillum rubrum-C*^{14}.

The description which follows applies to Figs. 2 to 5. At zero time the state of the several systems was as follows: (*a*) nuclei, 40 mg. protein with a specific activity of 1100 C.P.M. per mg. and 2200 C.P.M. of material soluble in TCA; (*b*) mitochondria, 30 mg. protein with a specific activity of 1560 C.P.M. per mg. and 3120 C.P.M. soluble material; (*c*) microsomes, 5 mg. protein with a specific activity of 1680 C.P.M. per mg. and 570 C.P.M. soluble material.

Incubations carried out under similar conditions to those given for Fig. 1, the medium for mitochondria being the same as that for microsomes. Symbols: nuclei, squares; mitochondria, triangles; microsomes, circles. Solid curve, no DNP added; broken curve, 10^{-4} M DNP present.

TABLE II

*Effect of pH during Incubation on the Release of Radioactivity from the Proteins of the Particulates from Livers of Rats**

	Radioactivity released, *counts/min.*	
	*p*H 7.2	*p*H 5.3
Nuclei	2440	5840
Mitochondria	2350	7080
Microsomes	787	1720

* Labelling of proteins, fractionation of tissues, and incubations (in O_2/CO_2) as in Table I.

In the experiments which have just been considered, amino acids were released from all fractions whether the conditions were aerobic or anaerobic, or whether DNP was present or not. It is possible that proteolytic enzymes were responsible. This possibility is further emphasized by the results of experiments

in which some of the incubations were carried out at pH 5.3. The data are shown in Table II, and demonstrate quite clearly that the release at pH 5.3 was two or three times greater than at pH 7.2. The same phenomenon was shown to occur in slices by Steinberg and Vaughan (2), and must be correlated with the observations that the optimum pH of most known tissue cathepsins is about pH 5 or lower (11, 12).

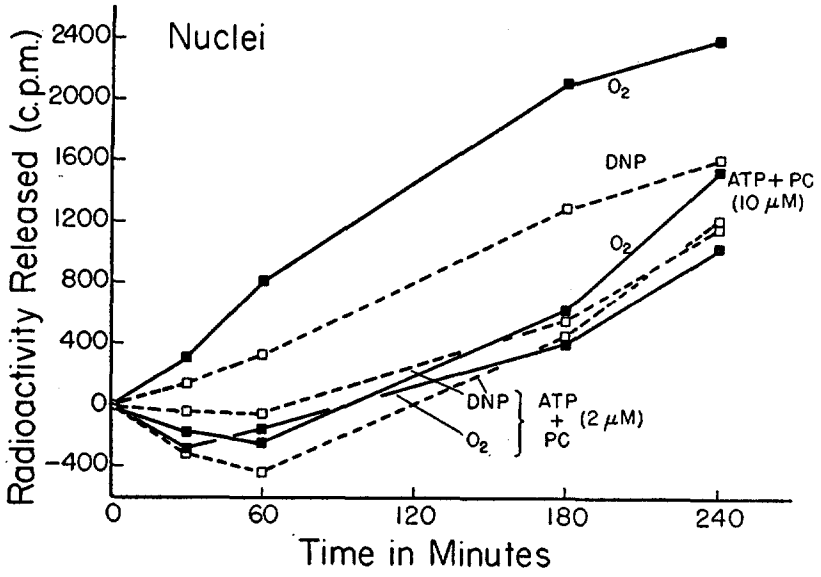

FIG. 3. Effect of conditions on "release" of label from nuclei isolated from livers of rats previously labelled by feeding *Rhodospirillum rubrum*-C^{14}.

Conditions as indicated in subscripts to Figs. 1 and 2.

It is of interest to note that the data in Table I show that the percentage of the activity released from the nuclei is greater than that from the microsomes, 8.7 per cent *versus* 3.7 per cent, the release from the mitochondria being between these values (7.8 per cent). Likewise, from the data given in Figs. 3 to 5, it can be shown that a similar situation prevails, the percentage release from the nuclei, mitochondria, and microsomes being 5.5, 4.6, and 1.6, respectively, in 4 hours in oxygen. The data from the sulfur-labelled particulates, plotted in Fig. 1, bear a similar relationship to each other. In 6 hours there was a release of 3.9 per cent of the activity from the nuclei compared with a 2.2 per cent release from the microsomes.

If the release phenomenon is to be associated with catheptic activity, then one would anticipate the greatest percentage of release from the particles bearing such activity. From the work of Gianetto and de Duve (13) and others

(14), it is apparent that catheptic activity is found in particles, the lysosomes, which are intermediate in size between the mitochondria and microsomes. The high rate of release from nuclei can hardly be accounted for on this basis unless the contamination with mitochondria is greater than we suppose. Some other factor or factors must be involved in the release phenomenon apart from catheptic activity.

It is not clear whether the considerable breakdown at pH 7.3 under anaerobic conditions, or when DNP is present, is brought about by cathepsins not at

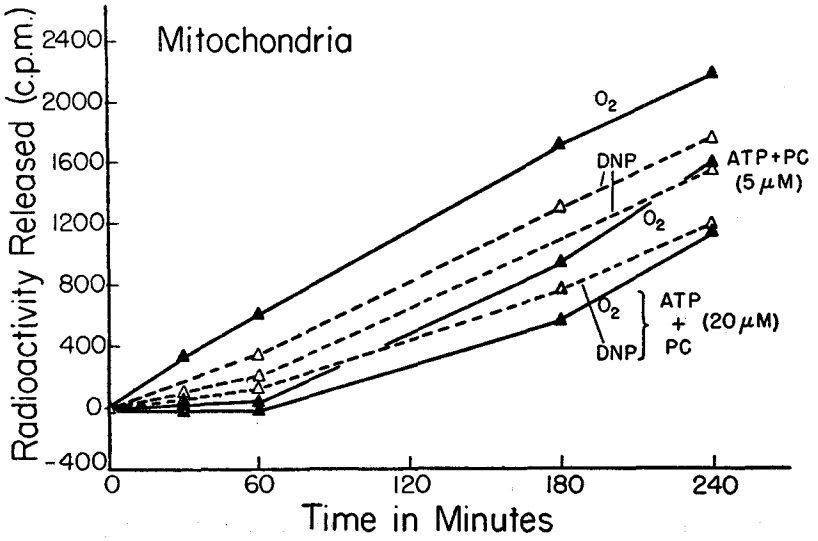

FIG. 4. Effect of conditions on "release" of label from mitochondria isolated from livers of rats previously labelled by feeding *Rhodospirillum rubrum*-C^{14}.

Conditions as indicated in subscripts to Figs. 1 and 2.

their optimum pH, or by cathepsins with a higher pH optimum than that usually found for these enzymes.

When ATP was added to the incubation medium, especially if PC or 3-phosphoglycerate was also present, the release of amino acid was depressed in all fractions. Indeed, in some experiments the initial level of non-protein radioactivity in the nuclear fractions was depressed after a short period of incubation (Figs. 3 and 5), particularly during the 1st hour. A similar but greater fall is shown by the microsomal fraction, but whereas the initial level in the mitochondrial fraction does not fall, it does remain almost constant during the 1st hour of incubation.

The uptake of radioactivity by the nuclear and microsomal fractions, that is the fall in counts, must represent a reincorporation of the amino acid into the

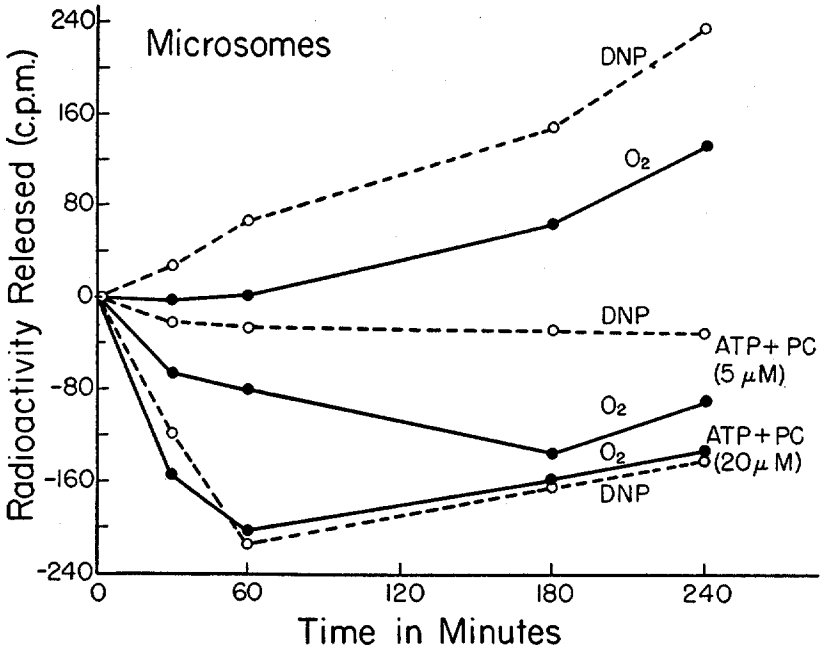

FIG. 5. Effect of conditions on "release" of label from microsomes isolated from livers of rats previously labelled by feeding *Rhodospirillum rubrum*-C^{14}.

Conditions as indicated in subscripts to Figs. 1 and 2.

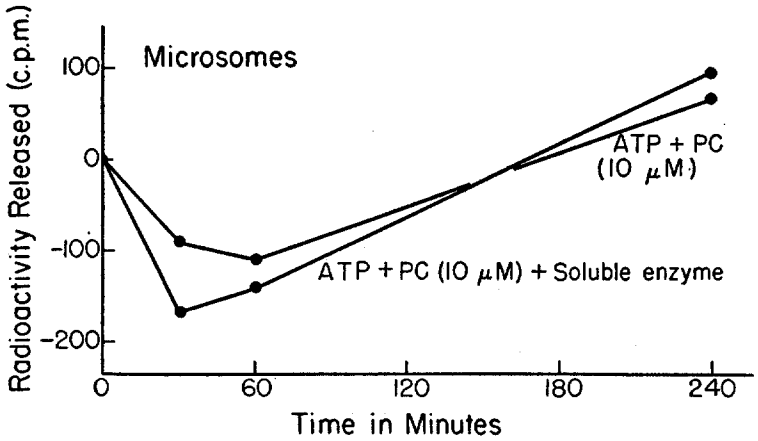

FIG. 6. Effect of activation on "release" of label from microsomes isolated from livers of rats previously labelled by feeding *Rhodospirillum rubrum*-C^{14}.

Conditions as in Figs. 1 and 2 except as noted below. The original state of the systems was as follows: microsomes, 7 mg. of protein with a specific activity of 585 C.P.M. per mg., and 485 C.P.M. in non-protein fraction. Some flasks contained the pH 5 activating enzyme prepared as described by Hoagland and coworkers (9). It was added in the amount of 5 mg. per sample in 0.1 M tris buffer.

protein. This reincorporation in the microsomes is increased by adding the soluble enzyme of Hoagland and coworkers (9), as shown by the data plotted in Fig. 6.

In Table III are shown the effects of omitting the carrier amino acids from the medium. Their absence results in a great depression of release during a 4 hour incubation, whether nuclei, mitochondria, or microsomes are being incubated. Similar results were obtained by Steinberg and Vaughan (2).

TABLE III

*Effect of Carrier Amino Acids on the Release of Radioactivity from the Proteins of the Particulate Preparations from Liver of Rats**

	Radioactivity released, *counts/min.*	
	Carrier amino‡ acids present	Carrier amino acids absent
Nuclei	986	102
Mitochondria	1040	196
Microsomes	672	72

* Labelling of proteins, fractionation of tissues, and incubations (under O_2/CO_2) as indicated in subscript to Table I.

‡ To each milliliter of medium were added 0.5 mg. of L-amino acids in the following percentages: arg-HCl, 6; his-HCl, 2; lys-HCl, 8; tyr, 4; ser, 7; leu, 8; ileu, 4; val, 6; glu, 13; asp, 6; gly, 5; ala, 5; pro, 5; h-pro, 1; cy-SH, 3; try, 2; phe, 5; met, 5; thr, 5.

DISCUSSION

The interpretation of the results obtained in this investigation and of those obtained by Simpson (1) and by Steinberg and Vaughan (2) depends on the assessment of three important unknowns in the system: (*a*) the rate of protein synthesis in the particulates (or slices); (*b*) the specific activity (SA) of the "free" amino acid pool in the particulates; (*c*) the effect of the conditions of incubation on the SA of the "free" amino acid pool in the particulates.

(*a*) *The Rate of Protein Synthesis.*—The data plotted in Fig. 5 show that in 1 hour approximately 213 counts/min. were taken up by the microsomes out of a total of 570 counts/min. in the non-protein fraction when ATP and PC were present (20 μM). If we assume for the moment that the SA of the amino acids in the protein is the same as the SA of those in the non-protein fraction then we can calculate the protein synthesized per hour as being $213/1680 = 0.127$ mg. when 1680 is the SA of the protein as found. The microsomes contained about 5 mg. of total protein, hence the fraction of the microsome protein replaced per day is $0.127 \times 24/5 = 0.61$. Or the half-life of the microsome protein is 0.693/0.61 or 1.1 days. Under conditions of stimulation with the soluble factor, the half-life of the microsome protein turns out to be 0.35 day.

In making these caluclations the SA of the "free" amino acid in the particles

was assumed to be the same as that in the protein. If we had made the superficially more reasonable assumption that the amino acid in the particles, the so-called "free" amino acid, had a lower specific activity due to dilution with the carrier amino acid, then the half-life of the microsomal protein would have been less than that calculated.

The half-life of the total liver protein has been variously estimated as being from 6 to 3.8 days (15–17). Consequently, it is to be anticipated that the microsomes will have a much shorter half-life than the lowest of these values, the uptake into microsomes from liver being more rapid than into other fractions (4). By calculation from the *in vivo* experimental data of Keller, Zamecnik, and Loftfield (17; see their Table 1), a half-life of 1.4 days is obtained. This value is probably too high because it is assumed in the calculation that the SA of leucine at the site of synthesis, in the microsomes, is the same as that of the injected dose. From the work of Loftfield and Harris (18) this is clearly not the case. Comparison of the half-lives of the microsomal protein from our *in vitro* experiments and the *in vivo* experments of Keller and coworkers (17) shows them to be comparable, both being overestimated. From this it necessarily follows that the specific activity of the "free" amino acid within the microsomes must be at least of the same order as that in the protein in spite of the addition of relatively large amounts of carrier amino acids.

The data make it clear that "free" amino acids are being incorporated into microsomal protein at the beginning of the incubation period under some conditions, and to a lesser extent this is true with the nuclei. It may therefore be stated with some confidence that the same phenomenon is also occurring in the mitochondria: in all three particulate systems it is probable that under most conditions employed, both synthesis and degradation of protein are occurring simultaneously. Depending on the conditions and system the net effect may be uptake or release of amino acid.

(*b*) *The SA of the "Free" Amino Acid Pool.*—It has been deduced that the "free" amino acid pool in the microsomes at least is not in rapid equilibrium with the carrier amino acids added to the medium, otherwise reincorporation could not have occurred. Consequently, these amino acids, which presumably arose by prior degradation of the microsomal protein, must have had a high specific activity. How can this be? Either these amino acids were bound to some structure in the microsomes by bonds which are very weak or easily hydrolyzed, or the amino acids were in some activated form, or there is an active system transferring amino acids into the particles. Some support is found for the first hypothesis in the work of Cohn and Rickenberg (19), Britten, Roberts, and French (20), and Cowie and Walton (21) with *Escherichia coli* and *Torulopsis utilis*. In those organisms before protein synthesis occurs, amino acids become bound to some part of their internal structure. There is also support for the second hypothesis in that various workers (22, 9, 23, to 25) have demonstrated

the presence of activated amino acids when incorporation is occurring. The third hypothesis appears plausible, but is not supported by any experimental work with which we are acquainted.

Therefore, there is ample reason to believe that the "free" amino acids in the microsomes and probably in the other particulates are not in rapid equilibrium with the carrier amino acid pool in the medium. A similar situation was observed by Loftfield and Harris (18), who failed to wash out radioactive amino acid from the pool in intact rats by infusing large amounts of inactive amino acids. Even more pertinent are the results of Hultin (26) and Hultin and Beskow (27) who showed that after a few minutes' incubation of labelled glycine or leucine with mitochondria, in a system similar to that described by Zamecnik and Keller (5), the addition of unlabelled glycine or leucine had no effect whatsoever on subsequent incorporation. In some of the experiments reported by Hendler (28), with respect to the labelling of the proteins in hen's oviduct tissue, the same type of phenomenon appears to have occurred.

It is also clear that the carrier amino acids do eventually have considerable effect on the SA because the release of radioactive amino acids from slices in 4 hours is much greater when carrier is added to the medium (1, 2). The same effect is noted with all three types of particulates (Table III). However, the carriers can only be partly effective in short term incubations. Since interpretation of the data of Simpson (1) and Steinberg and Vaughan (2) as a coupling of synthesis and degradation rests on the assumed effectiveness of the carrier addition, this interpretation may be incorrect. The probability that carrier is only partly effective is obviously even greater in slices than in particulate systems.

(*c*) *The Effect of the Conditions on the SA of the "Free" Amino Acid Pool in the Particles.*—It is clear that energy may be used to bind the amino acids in the particles, form "activated" amino acid, or transfer unlabelled amino acid into the particles, and thus promote the exchange of labelled amino acids. Alternatively, energy may be used to promote protein synthesis. Consequently, considering all the unknowns, the effects of adding ATP, DNP, etc., to the system of labelled particles—inactive amino acid can hardly be interpreted. The behavior of the radioactive release will depend on the dilution and washout of the radioactive amino acids from the particulates by the added carrier, and on the prevailing rate of synthesis. Moreover, the addition of amino acid analogs to the system does not lead to results which clarify the problem particularly. The analogs may well interfere with many of the processes just mentioned.

Superficially the demonstration by Steinberg and Vaughan (2) that there is an increase in non-protein nitrogen (NPN) in the system when slices are incubated, and that the rise in NPN under various conditions parallels the release in amino acids, supports the concept that degradation and synthesis are related phenomena. Again, however, in view of the complexity of the system, it is inadvisable to jump to conclusions.

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