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The Labelling by [^{14}C]Amino Acids of Cell-Sap Protein in a Cell-Free System from Guinea-pig Liver

THE SITE OF ORIGIN OF LABELLED PROTEIN

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It is now established that amino acids are incorporated into the protein of the microsome fraction of tissues such as liver at a more rapid rate than into the protein of other subcellular fractions both in the intact animal (e.g. Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950; Keller, 1951) and in cell-free systems (e.g. Siekevitz, 1952; Zamecnik & Keller, 1954). These findings suggest that the microsome fraction is of importance as a site of synthesis of other cytoplasmic proteins. While no evidence has been obtained showing that the microsome fraction is the site of origin of all cytoplasmic proteins, some evidence has been obtained which indicates that it is the site of synthesis of certain specific soluble proteins (e.g. Peters, 1957; Rabinovitz & Olson, 1956). It would be therefore of great value if the synthesis of specific soluble proteins could be studied in entirely cell-free systems. However, few instances have been reported of an unequivocal demonstration of either the labelling or net synthesis of a specific soluble protein in a cell-free system. In addition, a previous study made in this Laboratory (Simkin & Work, 1957*b*) has suggested that there might be some difference between the incorporation of amino acids into the protein of the microsome fraction of intact cells of guinea-pig liver and the incorporation into the microsome protein of a corresponding cell-free system. A microsome–cell-sap system of the type described by Zamecnik & Keller (1954) was used for these studies, and we reported that some soluble proteinaceous material present in the cell-sap fraction became labelled to a significant extent upon incubation. This finding prompted an investigation into the origin of the soluble protein which becomes labelled in this system. The present paper provides evidence suggesting that the microsome fraction does act as site of origin of the labelled soluble protein, and data are reported relating to factors affecting the passage of radioactivity from microsome to cell-sap fractions. This study forms part of a more general investigation into the significance of amino acid incorporation in cell-free systems, in the course of which it is hoped to discover whether specific soluble proteins are formed in such systems and whether the microsome fraction is the source of origin of such proteins.

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EXPERIMENTAL

Animals. As in earlier studies (Simkin & Work, 1957*b*), young guinea pigs (400–500 g.), which had been starved overnight, were used.

Materials. Reagents were prepared as described by Simkin & Work (1957*b*), except that in some experiments a purified preparation of [^{14}C]chlorella-protein hydrolysate was used (see below). Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) were purchased from the Sigma Chemical Co.

Purification of chlorella-protein hydrolysates. Hydrolysates of [^{14}C]chlorella protein were prepared as previously described. The amino acids were adsorbed on a column of Zeo-Karb 225 (H^+) and eluted with aq. 0·3 M-NH_3. The NH_3 was removed *in vacuo* and the residue dissolved in water. The use of such purified preparations resulted in slightly lower protein specific-activity values being obtained compared with values with chlorella-protein hydrolysates not subjected to this treatment. In other respects the results with both kinds of preparation were very

similar in studies both of incorporation into protein and of transfer of radioactivity from labelled microsome material. Zero-time values for cell-sap protein in incorporation studies were, however, lower when purified preparations were used. We have thus been unable to confirm, under the conditions employed in the present study, the finding of Craddock & Dalgliesh (1957) that purification of [^{14}C]-chlorella-protein hydrolysates is attended by a large reduction in the incorporation of radioactivity into protein.

Preparation of ^{14}C-labelled microsome fractions. Microsome–cell-sap preparations were prepared from 0·25 M-sucrose homogenates of liver as described by Simkin & Work (1957*b*). For the preparation of labelled microsome material, the microsome–cell-sap preparations were mixed with the same incubation medium as used earlier (for details, see Fig. 1), except that in some instances the concentration of [^{14}C]chlorella-protein hydrolysate was one-half of that used previously, although the total radioactivity added was the same as before. From data reported earlier (Simkin & Work, 1957*b*), it may be calculated that the amount of labelled amino acid added is only about 2·5% of the unlabelled amino acid already present in the system, and a reduction of the quantity of amino acid added should therefore make no appreciable difference to the total concentration. The conditions of incubation were those used previously. The total volume of the cell-free systems used was in general 14·4 ml. as before, but in some cases, where the labelled microsome material was not subsequently reincubated, the total volume was reduced to 3·6 ml. (in such cases, sucrose–casein hydrolysate was added as described below before centrifuging). After incubation, the mixtures were rapidly cooled in ice, and microsome and cell-sap fractions were separated by centrifuging without delay for 45 min. at 40 000 rev./min. ($g_{av.}$ 105 000) in Rotor 40 of a Spinco model L ultracentrifuge.

Preparation of cell-sap media for reincubation of microsomes. When cell-sap preparations were required for the reincubation of labelled microsome material, the microsome–cell-sap preparations (10 ml.) were mixed with the usual incubation medium (4·4 ml.), from which, as indicated in the Results section, [^{14}C]amino acids, 3-phosphoglycerate or $MgCl_2$ were omitted as required. Cell-sap fractions were obtained by centrifuging for 45 min. as described above, without prior incubation.

Reincubation of labelled microsome fractions. The surface of the labelled microsome pellet, prepared as described above, was washed twice with 2–3 ml. of 0·25 M-sucrose. The microsome material was then resuspended in a volume of cell sap (see above) equal to that from which it had been separated. Resuspension was effected in a Potter–Elvehjem-type homogenizer with a Teflon (polytetrafluoroethylene) pestle (Simkin & Work, 1957*a*) which was driven at about 1000 rev./min. Samples (3·5 ml.) of the suspensions were then incubated exactly as described earlier. Approximately 100 min. elapsed between the end of the first incubation (preparation of labelled microsomes) and the start of the second incubation (with fresh cell sap); during this time, all operations were carried out at 0–5°.

Separation of microsome and cell-sap fractions and isolation of protein for radioactivity measurement. At the end of incubation, approximately 2·5 vol. of an ice-cold 0·25 M-sucrose solution containing unlabelled amino acids (10 mM with respect to casein hydrolysate N) was added to the incubation mixtures. The diluted incubation mixtures were cooled in ice and then centrifuged without delay for 45 min. at 40 000 rev./min. The supernatants were removed as described earlier (Simkin & Work, 1957*a*) and taken as the cell-sap fractions. The surface of the microsome pellets was washed with 0·25 M-sucrose as described previously. Both microsome and cell-sap samples were frozen solid and stored in this state (usually at −35°) until taken for isolation of protein.

Microsome pellets were resuspended in iced water (5 ml.) by homogenization before precipitation of protein. Protein was precipitated with trichloroacetic acid (TCA), reprecipitated, purified and plated at infinite thickness as described by Simkin & Work (1957*a*).

Preparation of the trichloroacetic acid-soluble fraction of the cell sap for radioactivity measurement. The TCA-soluble fraction of the cell sap was prepared and diluted (tenfold) as described by Simkin & Work (1957*b*). A sample (0·5 ml.) was added to a 4·7 cm.^{2} nickel planchet which contained a few drops of diluted detergent and a disk of lens paper, and water was then removed *in vacuo*.

Determination of radioactivity. Samples were counted to an accuracy of at least ±5% with an end-window Geiger counter. Under the conditions used, 1 cm.^{2} of methacrylate resin of 10^{-3} μc/mg. gives 1000 counts/min. at infinite thickness.

RESULTS

Incorporation of [^{14}C]amino acids into microsome and cell-sap protein in a cell-free system

The time course of incorporation of a mixture of [^{14}C]amino acids into the protein of both microsome and cell-sap fractions in a microsome–cell-sap system from guinea-pig liver has been described (Simkin & Work, 1957*b*) (see also Fig. 1). There is an approximately linear increase in the specific activity of the microsome protein for about 20 min. The specific activity of the cell-sap protein increases only slightly during the first 10 min. of incubation, but then shows a rapid increase during the succeeding 10 min. period. After incorporation into microsome protein has apparently ceased, there is a further period during which the protein of the cell sap continues to increase in radioactivity. While the length of time during which amino acids are incorporated into microsome protein varies from preparation to preparation (20 ± 5 min.), the specific-activity curve for cell-sap protein always follows the specific-activity curve for microsome protein in an essentially similar manner to that shown in Fig. 1.

Because the amount of protein present in the cell-sap fraction is greater than that in the microsome fraction in this system (Simkin & Work, 1957*b*), it is preferable to plot data in terms of total amino acid uptake rather than as specific activity. The ratio of cell-sap protein to microsome protein is 3·8:1 ± S.D. 0·4 (16 preparations). The reason why this ratio is higher than that found when a more refined method of cell fractionation

(de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) is applied to a guinea-pig-liver homogenate is that losses of microsome material occur during the preparation of the microsome–cell-sap fractions. A comparison of the total number of counts in the protein of microsome and cell-sap fractions can be made by multiplying the specific-activity values of the cell-sap protein by the ratio of cell-sap protein to microsome protein; in the experiment shown in Fig. 1, this ratio was 4:1. When the results of a typical experiment are plotted in this manner (Fig. 1), it is seen that after 20 min. incubation the radioactivity of the cell-sap protein forms a substantial proportion (about 30 % in this case) of the radioactivity present in the protein of the system as a whole. Preliminary results indicate that a similar distribution of protein radioactivity is obtained with microsome–cell-sap preparations from rat liver.

Radioactivity of cell-sap protein after reincubation of labelled microsome fractions with unlabelled cell sap

In experiments such as that illustrated by Fig. 1, it is impossible to determine whether the radioactive protein appearing in the cell sap is derived from the microsome fraction or whether it is synthesized by a microsome-independent process. Further experiments were designed to clarify this point.

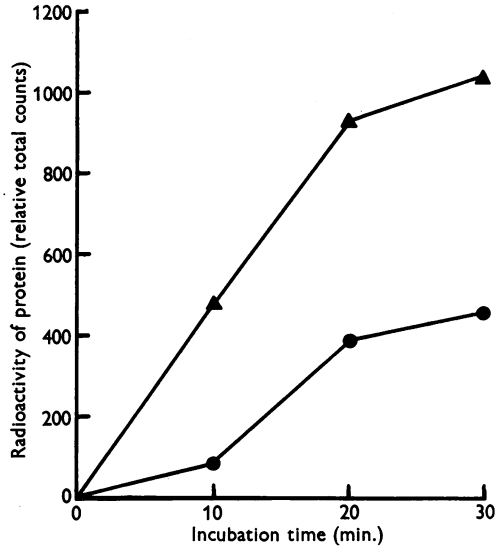

Fig. 1. Incorporation of [^{14}C]amino acids into microsome (▲) and cell-sap (●) protein upon incubation of a cell-free system prepared from guinea-pig liver. The incubation mixture contained: microsome + cell-sap fractions; KCl (20 mM); $KHCO_3$ (25 mM); potassium phosphate buffer, pH 7·8 (15 mM); $MgCl_2$ (2·5 mM); potassium 3-phosphoglycerate (10 mM); [^{14}C]chlorella-protein hydrolysate (0·09–0·18 mM, 0·8 μc/ml.). Incubation was carried out at 37° in an atmosphere of $N_2 + CO_2$ (95:5, v/v). For other details see text. The microsome values are plotted in terms of counts/min./cm.^{2} and the cell-sap values as 4 × counts/min./cm.^{2} (see text). The values have been corrected for zero-time uptake.

If cell sap was isolated from a microsome–cell-sap preparation, and then supplemented with [^{14}C]-amino acids, cofactors, etc., in the usual way and incubated, there was no significant incorporation of amino acid into the cell-sap protein. Alternatively, if a microsome–cell-sap preparation was incubated for 10 or 20 min. with [^{14}C]amino acids in the usual way and then centrifuged to remove microsomes, the cell sap thus obtained showed no further increase in the specific activity of its protein upon subsequent incubation. It thus seemed probable that the microsome material played an essential part in the formation of cell-sap protein in this cell-free system.

A direct test of this hypothesis could be made by incubation of [^{14}C]labelled microsome material with fresh cell sap. The protein-specific-activity curves of Fig. 1 show that after 10 min. incubation microsome protein is labelled to a substantial extent, whereas the cell-sap proteins contain little ^{14}C. It seemed likely therefore that microsome material isolated after 10 min. incubation in the presence of [^{14}C]amino acids would be particularly suitable for the study of transfer of radioactivity from microsomes to cell-sap protein, and such microsome material has been used throughout unless otherwise stated.

The basic plan of the experiments to be described was as follows. A microsome–cell-sap preparation was incubated with [^{14}C]amino acids in the usual way for 10 min. The incubation mixture was centrifuged, and the labelled microsomes so obtained were resuspended in unlabelled non-incubated cell sap. The latter was always prepared, as described in the Experimental section, from the same microsome–cell-sap preparation as that used to obtain the labelled microsomes, and in all cases contained the customary buffers and potassium chloride. When the cell sap used for resuspension was supplemented with 3-phosphoglycerate and Mg^{2+} ions, there was an increase in the specific activity of the cell-sap protein upon incubation under the customary conditions (Fig. 2). This was accompanied by a decrease in the specific activity of the microsome protein (changes in microsome-protein activity are considered more fully in a later section). It will be seen that the cell-sap protein increases in activity over a period of about 20 min., and thereafter little or no increase is found. With some preparations the increase in activity was approximately linear with time, but more often the increase during the first 10 min. of incubation was greater than that occurring during the succeeding 10 min. period.

If 3-phosphoglycerate, which serves as substrate for an ATP-generating system, was omitted from the cell-sap medium used for resuspension of labelled microsomes, the increase in the specific activity of the cell-sap protein was much smaller than in the corresponding control (Fig. 3). The effect of omission was less marked during the first 10 min. of incubation than during the succeeding 10 min. period. The specific-activity values for cell-sap protein isolated after 20 min. incubation in the absence of 3-phosphoglycerate were only 16, 23 and 24% of those found in its presence. Similarly, if Mg^{2+} ions were omitted from the incubation medium, the specific activity of cell-sap protein was lower than in controls (Fig. 4). The effect of omission of Mg^{2+} ions, though again less marked during the first 10 min. of incubation, was not as great as that resulting from the absence of 3-phosphoglycerate: specific-activity values for cell-sap protein after incubation for 20 min. without Mg^{2+} ions were 42, 51 and 58% of the controls with Mg^{2+}. In several experiments, labelled microsomes were incubated for 15 or 20 min. with fresh cell sap but without either 3-phosphoglycerate or Mg^{2+}. The cell sap was then separated (without dilution with sucrose–casein hydrolysate) and reincubated at once (in the absence of microsome material) with the customary concentration of either 3-phosphoglycerate or Mg^{2+} ions respectively. In neither case was there an increase in the specific activity of the cell-sap protein as a result of the final incubation. The results of a typical experiment of this type are shown in Fig. 5.

In several experiments microsome material was isolated after incubation with [^{14}C]amino acids for 20 min. instead of the customary 10 min. When the labelled microsomes were reincubated with fresh cell-sap media similar to those described above, it

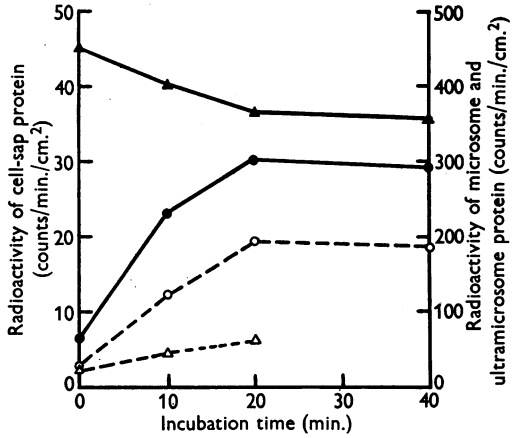

Fig. 2. Microsome material isolated after incubation for 10 min. with [^{14}C]amino acids, as described for Fig. 1, was reincubated with an unlabelled non-incubated cell-sap preparation (see text). The final concentrations of various additions and conditions of incubation were as for Fig. 1. After incubation, the cell-sap (●) and microsome (▲) fractions were separated and their protein isolated. Samples of the cell-sap fractions were also centrifuged at 105 000 $g_{av.}$ for 6 hr. before precipitation of protein to obtain recentrifuged cell-sap (○) and ultramicrosome (△) fractions. The ultramicrosome-protein value plotted at 20 min. is for material isolated from the pooled 20 and 40 min. samples

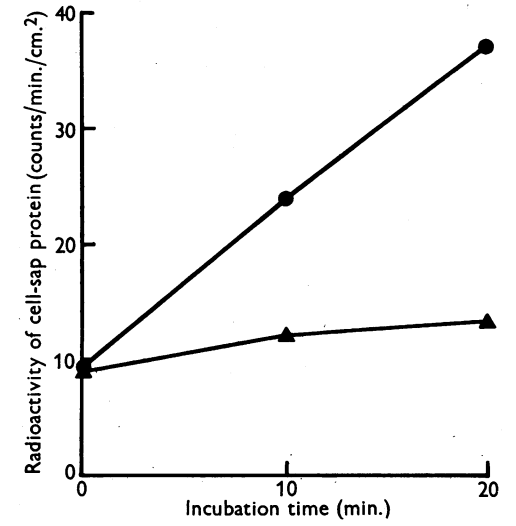

Fig. 3. Microsome material isolated after incubation with [^{14}C]amino acids for 10 min. was reincubated with a cell-sap preparation with (●) or without (▲) the addition of 3-phosphoglycerate. For details see Fig. 2.

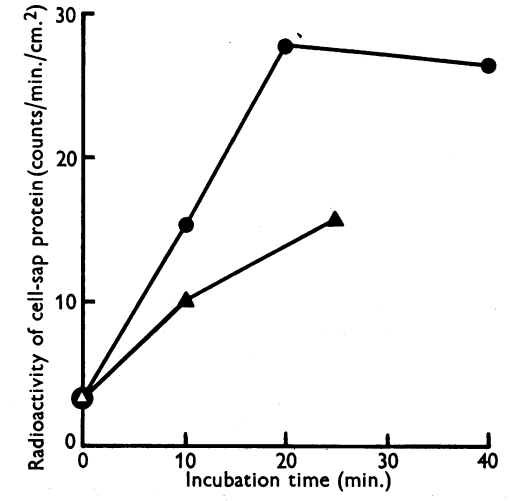

Fig. 4. Microsome material isolated after incubation with [^{14}C]amino acids for 10 min. was reincubated with a cell-sap preparation with (●) or without (▲) the addition of Mg^{2+}. For details see Fig. 2.

was found that again there was an increase in the specific activity of the cell-sap protein. The increase, which occurred mainly during the first 10 min. of incubation, was somewhat smaller than that obtained under similar conditions with the same microsome material labelled initially for only 10 min. When 3-phosphoglycerate was omitted from the reincubation medium, the specific activity of the cell-sap protein was again lower than in the control, being about 50 % of the control value with 20 min. periods of incubation. The effect of omission of 3-phosphoglycerate was thus less marked than with microsome material initially labelled for 10 min.

If samples of cell sap, obtained by centrifuging incubated or non-incubated mixtures of labelled microsomes and fresh cell sap, were dialysed extensively at 2° against 0·14M-sodium chloride–0·02M-tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer, pH 7·2, before precipitation of protein with TCA, there was no significant change in the specific activity of the protein isolated compared with undialysed controls. When similar samples of cell sap were recentrifuged at 105 000 $g_{av.}$ for 6 hr., there was some reduction in the specific activity of the protein isolated from the resulting supernatant compared with protein from the original material (Fig. 2), but at least 50 % of the total protein radioactivity remained in the supernatant, except for the zero-time sample when the proportion was somewhat less. Centrifuging in such a manner would be expected to sediment completely particles possessing the reported characteristics (see, for example, Petermann & Hamilton, 1957) of liver cytoplasmic-ribonucleoprotein particles. The brown-coloured pellet which sedimented under these conditions contained about 20 % of the protein present in the original cell sap. The specific activity of this ultramicrosomal protein, while somewhat higher than that of the protein not sedimenting, was much lower than that of the corresponding microsome protein (Fig. 2).

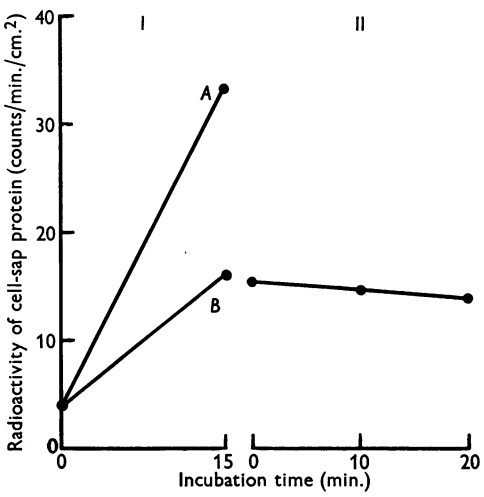

Fig. 5. I: microsome material isolated after incubation with [^{14}C]amino acids for 10 min. was reincubated with a cell-sap preparation with (*A*) or without (*B*) the addition of Mg^{2+} ions (for details see Fig. 2). II: cell sap which had been isolated from a separate sample of *B* after incubation was incubated (in the absence of microsomes) after the addition of Mg^{2+} ions to the usual final concentration.

Changes in the radioactivity of the protein of labelled microsomes upon reincubation

When labelled microsome material was reincubated with the customary cell-sap medium containing both 3-phosphoglycerate and Mg^{2+} ions, there was a decrease in the specific activity of the microsome protein. As a result of 20 min. incubation the mean fall in specific activity amounted to 22 % ± S.D. 4·0 (11 values) of the initial value. Because of the relatively small decrease in activity, it is difficult to determine with accuracy either the extent of the fall in activity or the nature of the change with time. However, in general the specific activity of the microsome protein decreased gradually for as long as the activity of the cell-sap protein increased, and then remained approximately constant (Fig. 2). The mean ratio, in terms of specific activity, of microsome protein decrease to cell-sap protein increase was 3·9:1 ± S.D. 0·9 (11 values, 20 min. incubation). If this ratio is calculated in terms of total counts rather than as specific activity as discussed earlier, using the mean value quoted above of 3·8:1 for the ratio of microsome protein to cell-sap protein, it will be apparent that there is an approximate equivalence between the decrease in microsome-protein radioactivity and increase in cell-sap-protein radioactivity. Because of the possible experimental errors, it cannot be established whether this equivalence is strictly held for each preparation.

There was some variation from preparation to preparation in the effects upon the specific activity of microsome protein of omission of 3-phosphoglycerate or Mg^{2+} from the media used to reincubate labelled microsomes. Thus, when 3-phosphoglycerate was absent, the decrease in specific activity was only 25–30 % of that found in the corresponding control in two preparations, but was similar to the value for the control in a third preparation. The percentage decrease in activity in the absence of Mg^{2+} was similar to that found for the corresponding control in two preparations, but in a third case the decrease was only about 50 % of that obtained in the control. If the small number of preparations studied and possible experimental errors are taken into consideration, no conclusions

can be drawn concerning the changes in the specific activity of microsome protein under these conditions.

The protein of microsome material incubated initially with [^{14}C]amino acids for 20 min. also decreased in specific activity upon reincubation with fresh cell sap. The percentage decrease was similar to that found with material labelled initially for 10 min. As the specific activity of the cell-sap protein was not very different in the two cases, the fall in microsome-protein radioactivity was thus larger than could be accounted for by the increase in that of cell-sap protein. With the one preparation studied, omission of 3-phosphoglycerate from the reincubation medium reduced the magnitude of the decrease in microsome-protein activity.

Factors other than experimental error might also play a part in producing variation in the level of microsome-protein radioactivity during reincubation. For example, radioactive protein could be degraded to TCA-soluble material, and there is in fact some evidence suggesting that this may occur. Thus, as previously reported (Simkin & Work, 1957*b*), there is a small increase of TCA-soluble amino N (as measured colorimetrically after reaction with β-naphthoquinonesulphonate) during incubation of the microsome–cell-sap system. Small changes in protein level may occur, but are not easy to detect. The fact that a small decrease in the specific activity of cell-sap protein has been found to occur after incorporation has ceased (see, for example, Figs. 2, 4 and 5) could be taken as evidence for protein degradation. The microsome–cell-sap fraction of liver is known also to contain some proteolytic enzymes (e.g. Rademaker & Soons, 1957).

Reincubation of labelled microsome fractions with fresh cell sap supplemented with [^{14}C]*amino acids*

A number of experiments were carried out to determine whether microsome material labelled with [^{14}C]amino acids for 10 min. in the usual way, and then isolated, was still capable of incorporating amino acids into its protein when resuspended in fresh cell sap and reincubated. These studies could also reveal whether it was possible for amino acids incorporated into microsome protein during reincubation to be transferred to the cell-sap protein. When labelled microsome material was resuspended in a fresh cell-sap medium supplemented with [^{14}C]amino acids, there was an increase in the specific activity of the protein of the microsome fraction in contradistinction to the fall observed in media without [^{14}C]amino acids. The increase in the specific activity of the cell-sap protein under these conditions was about twice that occurring in media not supplemented with [^{14}C]amino acids (Fig. 6). The additional increments in specific activity of both cell-sap and microsome fractions on reincubation in labelled media were, however, smaller (e.g. for cell sap, about one-half during the first 10 min. of incubation) than those obtained as a result of incubation of another sample of the original microsome–cell-sap system beyond the customary 10 min. period (Fig. 6). Systems containing microsome and cell-sap fractions separated and recombined without a preliminary incubation step have been found capable of incorporating amino acids into both microsome and cell-sap proteins at rates essentially similar to the corresponding system which had not been subjected to separation and recombination. This suggests that the decreased incorporation into protein found when labelled microsome material is reincubated may result from some kind of damage occurring during the preliminary incubation step.

It appeared possible that mixtures of labelled microsome material and fresh unlabelled cell sap might contain appreciable quantities of free [^{14}C]-amino acids which would be introduced into the system as a result of adsorption, etc., on microsomes during the initial labelling step. Some estimate of the extent of this contamination was obtained by comparing the TCA-soluble radioactivity of cell sap separated from mixtures of labelled microsomes and unlabelled cell sap with that of cell sap separated from the system used to prepare labelled microsomes. Some TCA-soluble radioactivity was found in cell sap from mixtures

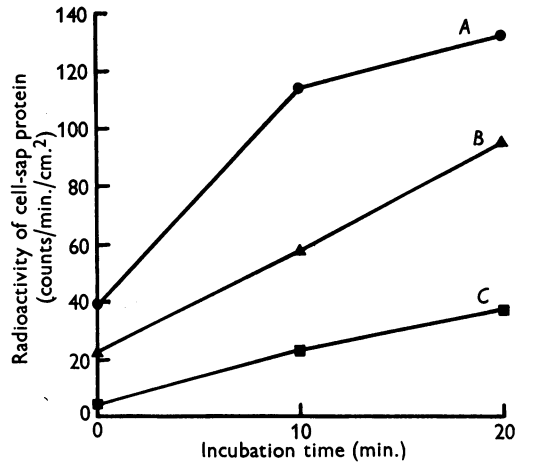

Fig. 6. A microsome–cell-sap preparation was incubated for 10 min. with [^{14}C]amino acids (see Fig. 1). One sample of the incubation mixture was then incubated further (*A*); zero time represents the beginning of the second period of incubation. Microsome material was isolated from a separate sample of the incubation mixture and reincubated with a fresh cell-sap preparation with (*B*) or without (*C*) the addition of [^{14}C]amino acids (for details see Fig. 2).

of labelled microsomes and unlabelled cell sap, but the amount present, though rather variable, was always small, being 2·7–6·6% (mean 4·0% ± S.D. 1·35, 10 experiments) of the quantity present in the original labelling system, and remained essentially constant during the customary period of incubation.

The effect of omission of either 3-phosphoglycerate or Mg^{2+} ions from reincubation media supplemented with [^{14}C]amino acids upon amino acid incorporation into protein has not been studied, but it has been found that omission of either from the original labelling system virtually abolishes incorporation.

Reincubation of labelled microsome fractions with sucrose-diluted cell-sap media

A study was made of the effect of replacing part of the cell-sap media used for the reincubation of labelled microsomes by 0·25 M-sucrose. Microsome material, labelled with [^{14}C]amino acids for 10 min. as before, was resuspended in a medium in which 75% of the cell sap had been replaced by 0·25 M-sucrose; 3-phosphoglycerate, Mg^{2+}, etc. were maintained at the usual final concentration. This medium was always supplemented (for the reason given below) with (in terms of final concentration) ATP (mM), GTP (0·15 mM) and additional Mg^{2+} (total, 5 mM), but despite this the increase in the radioactivity of the cell-sap protein after 20 min. incubation was, in terms of total number of counts in protein, only about one-quarter of the increase found for the corresponding control (i.e. undiluted cell-sap medium without ATP, GTP and additional Mg^{2+}). There was only a very small decrease in the specific activity of the microsome protein upon incubation with such diluted media, the decreases found after 20 min. incubation being only 13 and 25% of the decreases occurring in control media. In an experiment in which labelled microsomes were resuspended in a sucrose-diluted cell-sap medium supplemented with [^{14}C]amino acids (the amount of radioactivity added being one-quarter of normal in order to maintain the specific activity of the free amino acid pool approximately constant), it was found that amino acids were incorporated into the protein of both microsome and cell-sap fractions, but at rates much slower than those which were found with the control medium.

Investigations of amino acid uptake into microsome protein in systems in which non-incubated microsomes were resuspended in the sucrose–cell-sap medium had shown that, unless ATP and GTP were added, there was virtually no incorporation of amino acid into microsome protein (cf. Keller & Zamecnik, 1956). The addition of these two compounds and further Mg^{2+} ions (at concentrations given above) stimulated incorporation, but it has not proved possible so far to obtain a rate of incorporation similar to that of the control system (i.e. the customary labelling system). The restoration of incorporation into microsome protein brought about by these compounds has been somewhat variable: for 20 min. periods of incubation, values of 20, 37 and 63% of control incorporation were obtained with three separate microsome–cell-sap preparations.

DISCUSSION

The data reported support the hypothesis that in a microsome–cell-sap system the protein of the microsome fraction acts as precursor of some protein present in the cell sap. Significant labelling of cell-sap protein is found only when microsome material is present. When labelled microsome material is reincubated with unlabelled cell-sap preparations, the time course of the increase in the specific activity of cell-sap protein (e.g. Fig. 2) is essentially that which would be expected from the characteristics of amino acid incorporation into the original microsome–cell-sap system (e.g. Fig. 1). The decreases in the radioactivity of microsome protein obtained in a variety of unlabelled media have been found in most cases to be approximately equivalent to the increases in the radioactivity of the cell-sap protein; in the remaining instances the decreases found have been larger and not smaller than those expected. It seems reasonable to assume that a large proportion of the TCA-precipitable radioactive material present in the cell sap of the systems studied is in the form of soluble protein, for dialysis before precipitation does not affect the specific activity of the material, and centrifuging under conditions chosen to remove particles of the size of ribonucleoprotein granules and ferritin fails to sediment a substantial proportion of the TCA-precipitable radioactivity.

Although there appears to be transfer of label from microsome to cell-sap protein, it has not yet been unequivocally established whether label is transferred in the form of polypeptide or in the form of a relatively low-molecular-weight intermediate which would subsequently be polymerized in the cell sap. Such evidence as is available tends to favour the transfer of a large molecule. Free amino acids are excluded as intermediates in the transfer of radioactivity because no significant incorporation into protein occurs when cell sap, which contains appreciable quantities of free amino acids (about 7 mM in incubation mixture, Simkin & Work, 1957*b*), is incubated alone. Further, the possibility that the intermediate can be a compound in rapid equilibrium with the free amino acid pool is also excluded, because any such compound would be diluted very greatly if

released into unlabelled cell sap. Korner & Tarver (1957) have shown that microsome protein labelled *in vivo* can release under certain conditions small quantities of amino acid in a form that is not in rapid equilibrium with the free amino acid pool. Experiments already described (see, for example, Fig. 5) which were designed to test whether it is possible to show accumulation of such intermediates and their participation in the labelling of cell-sap protein gave negative results. Such experiments are not, however, conclusive because the release of intermediates from the microsome fraction might require the presence of 3-phosphoglycerate or Mg^{2+} ions; alternatively, the material released might not accumulate in the system.

The process resulting in the transfer of radioactivity from microsome to cell-sap protein appears to be an enzymic one, for omission of 3-phosphoglycerate, which would interfere with the generation of ATP, or of Mg^{2+} ions, markedly affects the process. The labelling of cell-sap protein which still takes place in media to which these compounds had not been added may, at least in part, be due to the presence of small quantities of the compounds which had been introduced by the labelled microsome material (cf. contamination with TCA-soluble radioactivity). The finding that substitution of 75% of the cell sap by 0·25M-sucrose reduces markedly the labelling of cell-sap protein may indicate that cell-sap enzymes are concerned with the process of passage of radioactivity from microsome to cell-sap protein; the reduction in labelling found might be due to interference with the generation of ATP, or perhaps to reduction in the concentration of enzymes or cofactors mediating in the transfer process. In one experiment it was found that replacement of the sucrose plus ATP and GTP used for dilution of cell sap by a dialysate of cell sap (of concentration equivalent to that normally present) did not result in an appreciable increase in the labelling of cell-sap protein. It is known, however, that incorporation of amino acid into protein is almost abolished by omission of either 3-phosphoglycerate or Mg^{2+} ions, and that it is reduced in sucrose-diluted cell-sap media. Therefore, the possibility cannot be entirely excluded that incorporation and transfer mechanisms are in some way obligatorily coupled to one another. It has been shown that amino acids are incorporated into microsome protein under the conditions used to study transfer of radioactivity.

The existence of a delay in the appearance of label in soluble protein presents an obvious analogy to the delay in the appearance of label in non-particulate specific proteins such as albumin (e.g. Peters, 1957), but there is as yet no evidence to show that the two phenomena are in fact related. The data reported by Peters are not adequate enough to establish clearly whether the lag period in the appearance of serum albumin in the medium is due merely to delay in excretion from the cell or in part due to the time required for release of albumin from the microsomes. The present results suggest that the latter might play some part, but it is unlikely that an adequate knowledge of this aspect of amino acid incorporation in cell-free systems will be obtained unless the labelling of specific proteins can be studied.

While this study was in progress, a number of reports appeared indicating that processes analogous to the one studied in the present investigation occur in other systems. Hendler (1957) reported that, if oviduct tissue was incubated with [^{14}C]amino acids and then homogenized, the ribonucleoprotein-containing cell debris so obtained appeared to release soluble radioactive protein. The transfer of radioactivity, which required ATP, took place under conditions in which there was no incorporation of amino acid into the ribonucleoprotein, and the radioactivity was present in protein fractions normally found in the cell sap. Rabinovitz & Olson (1957) also briefly reported similar results for reticulocytes; in this case the labelling of soluble haemoglobin was studied. Thus ribonucleoprotein labelled in intact cells can subsequently release soluble proteins under cell-free conditions, also by an energy-requiring process.

Campbell, Greengard & Kernot (1958) have recently reported that an albumin fraction bound to microsomes becomes labelled upon incubation of a microsome–cell-sap system from regenerating rat liver, but so far they have not reported the presence of label in albumin present in the cell sap. A failure to demonstrate the presence of labelled albumin in cell sap might be explained by interference with dissociation of albumin from microsome material resulting from cell damage. However, the studies of Hendler (1957) and Rabinovitz & Olson (1957) indicate that soluble proteins can be dissociated from ribonucleoprotein under cell-free conditions, at least with other tissues. The fact that in microsome–cell-sap systems there is an appreciable amount of label in cell-sap protein as a whole, but not in albumin, may indicate that it is not dissociation of protein from the microsomes that is interfered with, but rather that there is interference with the synthesis of specific proteins. Cell damage may disturb the normal pattern of protein synthesis; the differences in turnover of the protein of subfractions of microsomes labelled *in vivo* and in the cell-free system reported previously (Simkin & Work, 1957*b*) could be a reflexion of this. Thus while specific protein, such as albumin, might still be synthesized to some extent, protein synthesis might largely be redirected towards the synthesis of some non-specific or in-

complete protein. The failure to detect labelled albumin could, however, be the result of very great dilution with the pool of unlabelled albumin present in the system. It is hoped that the results of an investigation now in progress into the nature of the soluble protein labelled in the microsome–cell-sap system will clarify this point.

SUMMARY

1. When a microsome–cell-sap preparation from guinea-pig liver is incubated together with 3-phosphoglycerate, as adenosine triphosphate generator, and Mg^{2+} ions, there is incorporation of [^{14}C]amino acids into the protein of both microsome and cell-sap fractions. There is a delay of about 10 min. before appreciable label appears in the protein of the cell sap.

2. There is no significant incorporation under a variety of conditions of [^{14}C]amino acids into the protein of the cell sap in the absence of microsome material.

3. When microsome material is labelled by incubation with [^{14}C]amino acids for 10 min., then reincubated with fresh unlabelled cell sap, there is an increase in the specific activity of the cell-sap protein when the system is supplemented with 3-phosphoglycerate and Mg^{2+} ions; this is accompanied by an approximately equivalent decrease in the radioactivity of the microsome protein. The evidence obtained suggests that there is transfer of radioactivity from microsome to cell-sap protein.

4. In the absence of 3-phosphoglycerate, there is a marked reduction in the extent of labelling of cell-sap protein, indicating that an adenosine triphosphate-dependent reaction is associated with the transfer of radioactivity from microsome material. Absence of Mg^{2+} ions also reduces the labelling of cell-sap protein. Replacement of 75% of the cell sap by 0·25 M-sucrose reduces the transfer of radioactivity from labelled microsomes, despite supplementation with adenosine triphosphate and guanosine triphosphate.

5. Microsome material labelled by incubation with [^{14}C]amino acids for 10 min. is still able to incorporate amino acids into its protein when resuspended in fresh cell sap, although at a somewhat reduced rate.

6. The labelled material present in the cell sap appears to be largely in the form of soluble protein, and the possible nature of this material is discussed.

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The Relationship of the Tricarboxylic Acid Cycle to the Synthesis of δ-Aminolaevulic Acid in Avian Erythrocyte Preparations

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Shemin & Russell (1953) showed conclusively by ^{15}N- and ^{14}C-labelling experiments that δ-aminolaevulic acid incubated with duck blood was incorporated into the haem formed. Confirmation of δ-aminolaevulic acid as a porphyrin precursor in lysed red-cell systems was forthcoming from Neuberger & Scott (1953) and Dresel & Falk (1953).

It was postulated by Shemin & Kumin (1952) that succinyl-coenzyme A was involved in porphyrin synthesis and, upon the recognition of δ-aminolaevulic acid as a porphyrin precursor, it was suggested that succinyl-coenzyme A coupled with glycine to form δ-aminolaevulic acid via the unstable α-amino-β-oxoadipic acid (Shemin & Russell, 1953).