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The Incorporation of Radioactive Amino Acids into Proteins of the Microsome Fraction of Guinea-Pig Liver at very Short Time Intervals after Administration

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The question whether the process of incorporation of labelled amino acids into trichloroacetic acid-precipitable protein, which takes place in cell-free systems, represents part or all of the process resulting in the synthesis of proteins in the corresponding intact cell is an important problem in the interpretation of the results of many investigations into the mechanism of protein biosynthesis. Simkin & Work (1957*a*, *b*) attempted to obtain information on this question with respect to the microsome fraction of guinea-pig liver. In that work the microsome fraction was labelled with [^{14}C]amino acids by allowing incorporation to take place either in the intact guinea pig or in a cell-free system of the type described by Zamecnik & Keller (1954) which contains microsome and cell-sap fractions. The microsome fraction was then isolated, and separated into a series of distinct subfractions by a procedure involving successive extraction with solutions of varied ionic strength and pH. The radioactivity of the protein of the subfractions was determined. When the results of labelling under the two sets of conditions were compared, the pattern of labelling found in the cell-free system appeared to be different from that resulting from incorporation in the intact cell. The difference in behaviour was particularly noticeable in two ribonucleic acid-rich subfractions, *B* and *C*. The reasons for this difference in the labelling pattern were not clear. The shortest period of exposure to radioactive amino acids *in vivo* in this work was 2 min., and, as Simkin (1959) has pointed out, the possibility could not therefore be excluded that the pattern of labelling found in the cell-free system resembled that occurring in the intact liver cell as a result of periods of incorporation less than this. The results of analogous investigations carried out by Sachs (1958) lent support to this view. In order to resolve this question, the labelling of the microsome subfractions of guinea-pig liver occurring *in vivo* has been re-examined, very brief periods of exposure to [^{14}C]amino acids now being employed. The results obtained support the suggestion made above: there is a similarity in the pattern of labelling occurring in

the cell-free system to that found as a result of a period of incorporation of about 30 sec. in the intact liver cell. Some reinterpretation of our earlier work is therefore necessary.

EXPERIMENTAL

Animals. Guinea pigs were purchased from A. J. Tuck and Son, Rayleigh, Essex. The animals, of 630–750 g. body wt., were starved overnight before use.

Radioactive amino acids. A mixture of uniformly labelled L-[^{14}C]amino acids was prepared by acid hydrolysis of *Chlorella* [^{14}C]protein and purified by passage through a column of Zeo-Karb 225 as described by Simkin (1958). The solution used for injection contained amino acids (100 μc/ml.; 5 μmoles of N/ml.). in 0·9% sodium chloride.

Administration of radioactive amino acids and removal of liver. The animals were anaesthetized by combined intraperitoneal injection of sodium pentobarbitone (37–47 mg./kg. body wt., in the form of 0·40–0·55 ml. of oven Veterinary Nembutal, Abbott Laboratories, London) and inhalation of ether. The abdomen was opened and ligatures were loosely tied around the posterior vena cava anterior to the liver and around the portal vein. The purpose of the ligatures was to interrupt a major part of the supply of blood to the liver after the appropriate period of incorporation had been allowed to take place, in an effort to slow down any incorporation of amino acids which might occur during excision of the liver (cf. Loftfield & Eigner, 1958). In addition, the ligatures facilitated rapid removal of the liver. The [^{14}C]amino acid solution was injected into the portal vein about 2 cm. from the liver; the quantities of isotope administered were 80, 75 and 63 μc/kg. body wt. in Expts. 1, 2 and 3, respectively. After the appropriate time interval had elapsed, the ligatures were pulled tight, and the liver removed, immersed in a bath of ice-cold 0·25 M-sucrose, and immediately cut into pieces with scissors. The whole operation was carried out as rapidly as possible, and efforts were made to keep the animals warm.

Isolation and fractionation of microsome material. The procedure used followed closely that adopted in earlier work. In brief, the livers were fractionated by differential centrifuging as described by Simkin & Work (1957*a*). The microsome material so obtained was washed and fractionated by the technique employed for material which had been labelled in a cell-free system (Simkin & Work, 1957*b*); this method represents a slight modification of the original technique (Simkin & Work, 1957*a*). The fractionations were checked by determining the ultraviolet-absorption spectra of the microsome subfractions (Simkin & Work, 1957*a*).

Isolation of protein for measurement of radioactivity and determination of radioactivity. The methods employed were those detailed by Simkin & Work (1957*a*). In summary, after addition of a mixture of non-radioactive amino acids, protein was precipitated from samples by addition of trichloroacetic acid. The precipitates were dissolved in alkali in the presence of non-radioactive amino acids and protein again precipitated by the addition of trichloroacetic acid; the precipitates were then washed in order to remove nucleic acid and lipid. Radioactivity was determined with a thin end-window counter. With few exceptions, the standard deviation of the net count rate was not greater than $\pm 5\%$, and more often was ± 1 or 2%. With a few samples of very low specific activity, for practical reasons the standard deviation was greater, the highest value being $\pm 13\%$ (subfraction *A*, Expt. 2). Under the conditions of counting used, 1 cm.^{2} of methacrylate resin of 10^{-3} μc/mg. gives 1000 counts/min. at infinite thickness.

RESULTS

The results are presented of three experiments on the incorporation *in vivo* of [^{14}C]amino acids (*Chlorella* protein hydrolysate) into protein of subfractions of the microsome material of guinea-pig liver. In two experiments, (1 and 2), incorporation was allowed to take place for periods of time, namely 0·4 and 0·75 min., which were shorter than any used in the earlier investigation of Simkin & Work (1957*a*). The other experiment (3), was designed to determine whether the surgical procedure or anaesthetic used in the present work affected the pattern of labelling obtained, and in this instance a period of incorporation was employed, viz. 3·5 min., which lay between the two shortest time intervals which had been used in the earlier study in order to facilitate comparison with the results obtained in that work. The time intervals stated represent the time elapsing from the beginning of the injection of [^{14}C]amino acids into the portal vein to the time when the ligatures around the posterior vena cava and portal vein (see Experimental section) were pulled tight. The injection of the amino acid solution was completed within 6, 4 and 2 sec. in Expts. 1 2 and 3, respectively. After the interruption of the blood supply to the liver, a period of 9–10 sec. was required for excision of the liver and transfer to ice-cold 0·25 M-sucrose.

The results (Fig. 1) are given, as in earlier papers (Simkin & Work, 1957*a*, *b*), in terms of the ratio of the specific radioactivity of the protein of a microsome subfraction to the specific radioactivity of the protein of the whole microsome fraction at the corresponding time interval. This method of expression facilitates comparison of data obtained in different experiments. The actual specific radioactivities of the protein of the whole microsome fractions in Expts. 1, 2, and 3 were 43, 182 and 706 counts/min./cm.^{2} at infinite thickness, respectively. Fig. 1 shows that at short time intervals after injection of [^{14}C]amino acids, the protein of subfraction *A* (a fraction extracted by 0·14 M-NaCl, and containing mainly protein) possessed only a very low level of radioactivity when compared with the protein of the microsome fraction as a whole. After the shortest period of incorporation (about 0·4 min.), the protein of the ribonucleic acid (RNA)-rich subfraction *B*, isolated by extraction with M-NaCl, had a specific activity over twice as great as that possessed by the whole microsome protein. As the period of exposure to isotope was increased, however, the specific activity relative to that of the whole microsome protein decreased markedly. In

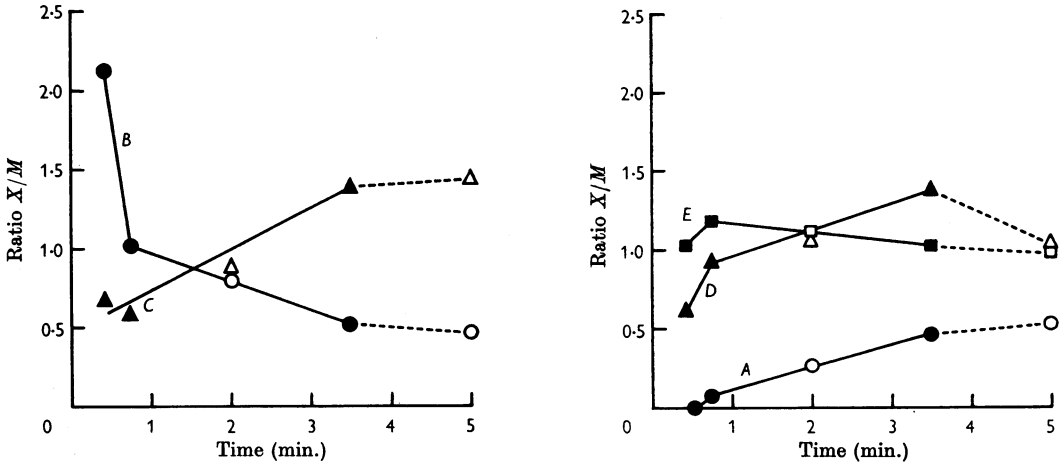

Fig. 1. Change with time of the radioactivity of proteins of subfractions (*A–E*) of microsome material from guinea-pig liver after injection of [^{14}C]amino acids. Radioactivity is expressed as the ratio of the specific activity of the protein of the subfraction (*X*) to that of the corresponding whole microsome protein (*M*). The values represented by the open symbols are those obtained in previous work (Simkin & Work, 1957*a*) in which unanaesthetized animals, not subjected to surgical disturbance, were used.

contrast, after either 0·4 or 0·75 min. incorporation, the specific activity of the protein of subfraction *C*, another RNA-containing fraction, extracted by 0·1 M-bicarbonate–carbonate buffer, pH 9·0, was about two-thirds that of the corresponding whole microsome protein, but the relative activity of the protein of *C* increased appreciably as a result of longer periods of incorporation. The proteins of the lipid-containing subfractions *D* and *E*, separated by means of their differing solubility in 0·01 N-NaOH, showed a slightly different behaviour to one another. The protein of *D* had at first a lower activity than that of the whole microsome protein, but later possessed an activity equal to, or slightly greater than, the whole. The protein of *E* possessed a rather similar specific activity to the whole microsome protein at each time studied.

The data presented in Fig. 1 indicate that there is excellent agreement between the results of Expt. 3 of the present investigation (3·5 min. incorporation) and the values which might be expected on the basis of earlier work for the incorporation pattern resulting from such a period of labelling. It therefore seems very probable that the surgical procedure, anaesthetic, or different route of administration of amino acid used in the present work do not affect the pattern of labelling obtained to any appreciable extent.

DISCUSSION

The results of the present investigation, when combined with the data obtained in earlier work (Simkin & Work, 1957*a*), indicate that the pattern of incorporation of amino acids into the protein of subfractions of guinea-pig-liver microsome material is constantly changing as the time interval after administration of labelled amino acid increases. With subfractions *A*, *B*, *C* and probably *D*, there is an increase in the value of the ratio of specific activity of subfraction protein to specific activity of corresponding whole microsome protein, followed by a decrease in the magnitude of the ratio. The proteins of the subfractions of high RNA content, *B* and *C*, exhibit this kind of behaviour to the most marked extent: in both instances, the maximum value for the ratio exceeds 2·0, the maximum for *B* being 0·5 min. or less after administration of amino acid and that for *C* about 30 min. later. The reasons underlying the complexity of the labelling of the microsome proteins remain obscure. The very rapid labelling of the protein of subfraction *B* was not detected in our earlier work since the shortest time period of incorporation investigated was 2 min. On the basis of the results then obtained, it was suggested that material contained in subfraction *C* might be of importance in the process of amino acid incorporation since the protein of *C* attained a particularly high level of radioactivity. There appeared to be an analogy with the behaviour of RNA-containing subfractions of other kinds isolated from rat-liver microsome material by a number of investigators (for some references, see Simkin & Work, 1957*a*), with the exception of the fact that the protein of other ribonucleoprotein fractions gained and lost radioactive amino acids at a much more rapid rate than did the protein of subfraction *C*. Thus, when Littlefield, Keller, Gross & Zamecnik (1955) studied the incorporation of [^{14}C]amino acids into the ribosomes of rat-liver microsome material, they found that the maximum specific

activity of the ribosome protein was reached 3 min. or less after the administration of amino acid. Much subsequent work has supported the suggestion of Littlefield *et al.* (1955) that the ribosomes are the primary site of polymerization of amino acids in the cytoplasmic material comprising the microsome fraction. It is now clear that it is the protein of the other RNA-rich subfraction, *B*, that behaves in an analogous way to that of ribosome protein and may therefore, at least in part, be derived from it. It is perhaps significant that the protein of subfraction *B* has a high content of arginine (Simkin & Work, 1957*b*) as, characteristically, has the ribosome protein of rat liver (Crampton & Petermann, 1959).

When the results of the application of the fractionation procedure to guinea-pig-liver microsome material labelled in a cell-free system (Simkin & Work, 1957*b*) are compared with those obtained in the present investigation, there is in fact a substantial degree of similarity between the pattern produced in the cell-free system as a result of about 15 min. of incubation and that which results from about 0·4 min. of exposure to labelled amino acid *in vivo*. The results are particularly striking for subfractions *B* and *C*: in either case, the protein of *B* has a specific activity over twice as great as that of the whole microsome protein, whereas the protein of *C* has a specific activity about 60–70 % of that of the whole protein. If incorporation is prolonged beyond this stage, the activity, relative to the total protein, of the protein of *B* decreases whereas that of *C* increases. With the cell-free system, however, incorporation into microsome protein as a whole ceases before this second stage is far advanced. There is also a general similarity in the behaviour of the proteins of subfractions *A*, *D* and *E* in the cell-free system and in the early stages of incorporation *in vivo*. It thus seems possible that the pattern of labelling in the cell-free system might result from the operation of a mechanism of amino acid incorporation which is possessed by the intact liver cell. On this view, the mechanism would operate in the cell-free system in an essentially similar manner to normal but at a much lower rate, i.e. about one-twentyfifth to one-fiftieth of that in the intact cell. The results of Sachs (1958), who used a different fractionation procedure to examine rat-liver microsome material labelled *in vivo* or in a cell-free system, are, where comparison can be made, in general agreement with those considered here. The findings of a number of recent investigations (e.g. Campbell, Greengard & Kernot, 1960) into protein synthesis, in cell-free systems of the type considered above, are consistent with the view that the process of incorporation in such systems represents, at least in part, the process resulting in the synthesis of protein in the intact cell. It is only by the study of the synthesis of specific proteins in cell-free systems that any definitive answer to the question of the validity of the use of such systems can be obtained.

SUMMARY

1. In extension of previous work (Simkin & Work, 1957*a*), guinea pigs were killed at short time intervals after the intraportal injection of a mixture of [^{14}C]amino acids (*Chlorella* protein hydrolysate). The microsome fraction of liver was isolated, and separated into a number of subfractions of distinct composition by a procedure involving successive extraction with solutions of varied ionic strength and pH.

2. The patterns of labelling of the protein of the microsome subfractions found after periods of incorporation of approx. 0·4 or 0·75 min. were different from those resulting from longer periods of incorporation and which have been reported earlier. In particular, the protein of the ribonucleic acid-rich subfraction *B* had a specific activity over twice that of the microsome proteins as a whole after 0·4 min. incorporation; this high relative value fell markedly, however, as the period of exposure to labelled amino acid was increased.

3. Earlier studies (Simkin & Work, 1957*a*, *b*) of the pattern of labelling of the protein of microsome subfractions, resulting from incorporation either *in vivo* or in a cell-free system, have been reinterpreted in the light of the present findings. The incorporation of amino acids into microsome protein proceeded for only about 20 min. in the cell-free system used, and the pattern of labelling found at that time is seen to be essentially similar to that produced by about 0·5 min. of exposure to labelled amino acid *in vivo*. The implications of this finding are discussed.

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