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The Reactions of Amino Acids with Soluble Ribonucleic Acid from Guinea-Pig Mammary Cells

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During the last 5 years many cell-free systems of animal, plant and bacterial origin have been prepared which were able to perform some of the intermediate reactions which are likely to be on the pathway to protein synthesis. The mammary gland in full lactation is a most suitable system for the study of every aspect of protein synthesis; not only are large quantities of protein continuously formed but they are also excreted from the cells and thus the complications introduced by catabolism are avoided. Barry (1958) has described various studies of the formation of milk proteins from injected labelled amino acids *in vivo*. He concluded that milk proteins are formed from free amino acids of the blood stream. The development of cell-free preparations from liver, which could incorporate [¹⁴C]amino acids into the peptide linkage of microsomal protein (Zamecnik & Keller, 1954; Keller & Zamecnik, 1956), led to the discovery of three distinct steps in this process (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958). The three steps are: first, the activation of amino acids by the enzymic formation of amino acid adenylates; secondly, the combination of activated amino acids with cell-sap ribonucleic acid; and thirdly the transfer of amino acids from cell-sap ribonucleic acid to microsomal nucleoprotein.

In this Laboratory we have studied many

aspects of amino acid incorporation with a number of different systems prepared from lactating mammary glands. The pattern of incorporation of amino acids into subcellular particles and soluble protein of intact mammary cells and tissue homogenates *in vitro* has been described recently (Fraser & Gutfreund, 1958), and we have shown that the three steps studied by Hoagland *et al.* (1958) can be usefully investigated in mammary-tissue homogenates. We have now studied the formation of cell-sap ribonucleic acid-amino acid compounds in greater detail. Our results do not enable us to draw any conclusions about the chemical mechanism of this reaction, but they give a quantitative picture of the proportions of different amino acids involved, of their competition with each other and of the rates and equilibria of the compound formation. It will be seen that certain conclusions can be drawn from such data about the role of cell-sap ribonucleic acid-amino acid coupling in the overall process of protein synthesis.

EXPERIMENTAL

Materials. All [¹⁴C]amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Their specific activities are quoted in relevant places in the text. The L-amino acids are generally labelled, and the DL-amino acids are [1-¹⁴C]. The adenosine triphosphate (ATP) used

was obtained from L. Light and Co. Ltd., Colnbrook, Bucks, as the free acid and converted into the magnesium dipotassium salt (MgK_2ATP).

Preparation of cell fractions. Guinea pigs, within 2–7 days after parturition, were killed by a blow on the head. Their mammary glands were excised and plunged into ice-cold medium A (containing 0.35 M-sucrose, 0.035 M- KHCO_3 , 0.025 M-KCl, 0.01 M- MgCl_2 ; pH 7.4), after Keller & Zamecnik (1956). After cooling for 3–4 min. the glands were dried and freed of extraneous fat, muscle and connective tissue and minced. The mince was weighed and 2.3 vol./wt. of ice-cold medium A added with stirring. It is important to keep the tissue cold in any operation subsequent to excision of the glands. The mince suspension was then homogenized in a Potter homogenizer kept in ice. The cell fractions were separated by differential centrifuging in a refrigerated Spinco preparative ultracentrifuge at 0°. An initial spin for 10 min. at 10 000 g sedimented the debris, nuclei and mitochondrial fractions. The 'microsome-rich fraction' thus obtained was then diluted 1:1 (v/v) with ice-cold medium A. A second spin for 90–120 min. at 105 000 g sedimented the microsome fraction, leaving a clear-pink supernatant fraction, which was carefully pipetted off.

Preparation of the 'pH 5 fraction' and cell-sap ribonucleic acid. The supernatant fraction from the differential centrifuging was further diluted 1:1 (v/v) with ice-cold 0.15 M-KCl and adjusted to pH 4.9 with N-acetic acid. The solution became turbid and the precipitate, the 'pH 5 fraction', was spun down, washed once by resuspending in ice-cold 0.15 M-KCl and again centrifuged. The final precipitate was then redissolved ready for use in a volume of medium A equal to the weight of the minced tissue used. In this way we obtained a suspension of 'pH 5 fraction' which contained 10–15 mg. of protein/ml. and 0.3–0.4 mg. of cell-sap ribonucleic acid (RNA)/ml. From this pH 5 fraction cell-sap RNA was prepared by the phenol-extraction procedure of Kirby (1956) as modified by Hoagland *et al.* (1958).

Estimation of cell-sap ribonucleic acid. The concentration of cell-sap RNA in the pH 5 fraction was determined after the method of Hoagland *et al.* (1958), by digestion of a sample in N-NaOH for 1 hr. at 37°, precipitation of the protein with 5 N-HCl and determination of the extinction at 260 μ with a Unicam model SP. 500 spectrophotometer. An extinction coefficient of 34.2 $\text{mg.}^{-1} \text{cm.}^2$ was used to calculate the cell-sap RNA concentration.

Procedure for labelling cell-sap ribonucleic acid by [^{14}C]amino acid-cell-sap ribonucleic acid coupling

Labelling experiments. Incubation mixtures contained MgK_2ATP , 'pH 5 fraction' dissolved in medium A (see above), medium A (with or without dissolved carrier or competitor amino acids) and [^{14}C]amino acid, in the ratio of 2:10:7:1 (by vol.), the final concentrations of ATP and [^{14}C]amino acid being respectively 0.01 M and 0.5 $\mu\text{C}/\text{ml.}$, except where stated. The total volume was adjusted according to the needs of the experiment. Incubations were carried out aerobically in a water bath at 37°. At appropriate times samples (1 or 2 ml.) were taken and precipitated in 5 ml. of ice-cold 10% trichloroacetic acid containing 1% of carrier amino acid. Either the total pH 5 fraction (cell-sap RNA + protein) or the pH 5 protein was then isolated for counting.

'Delabelling' experiments. The pH 5 fraction was prelabelled as above for 30 min. The reaction was stopped by plunging the flask into an ice bath with efficient stirring of the reaction mixture. The pH 5 fraction was then precipitated again by adjusting the pH to 4.9 with N-acetic acid and washed with ice-cold 0.15 M-KCl. The labelled pH 5 fraction was redissolved in the original volume of fresh cold medium A, with appropriate amounts of added MgK_2ATP , as described. Further incubation was then carried out for 'delabelling' at 37°, taking appropriate time samples (1 or 2 ml.) for precipitation in ice-cold 10% trichloroacetic acid containing 1% of carrier amino acid.

Preparation of samples for counting

The total pH 5 fraction (cell-sap RNA + protein) was isolated from the trichloroacetic acid precipitates (see above) by a slight modification of the method of Hoagland *et al.* (1958). The precipitates were washed twice with cold 5% trichloroacetic acid containing 1% of carrier, and once each with 80% ethanol, acetone, absolute ethanol-ether (3:1, v/v) and ether, in that order. The samples were then air-dried for counting. Cell-sap RNA as well as [^{14}C]amino acid-labelled cell-sap RNA samples were prepared in the same way.

Determination of radioactivity

All samples were counted at infinite thickness, on 0.3 cm.^2 or 1.0 cm.^2 plastic planchets. A [^{14}C]Perspex standard (supplied by The Radiochemical Centre) was used for frequent standardization of the counter. All samples were counted on a Tracer-Lab internal gas-flow counter. The background count varied from 16 to 18 counts/min. All counts are recorded as counts/min./ cm.^2 at infinite thickness, corrected for background. From self-absorption experiments carried out over a wide range of sample weights the relation $\text{counts/min./mg.} = \text{counts/min./cm.}^2$ (infinite thickness)/2.2 was established.

RESULTS

Apart from the previously noted isolation of [^{14}C]amino acid-labelled cell-sap RNA two types of experiment were carried out to show that the significant part of the ^{14}C -labelling of pH 5 fraction on incubation with ATP and [^{14}C]amino acids is due to the coupling of amino acids to the cell-sap RNA of this fraction. Fig. 1 shows the time course of the combination of [^{14}C]leucine with total pH 5 fraction and with protein prepared from this fraction. As indicated in the section above there is about 30 times as much protein as nucleic acid in the pH 5 fraction. We have confirmed the requirement for ATP for this reaction in the mammary-gland system, which has been reported by Hoagland *et al.* (1958) for experiments with rat-liver pH 5 fraction.

Next we investigated the effect of amino acid uptake into the pH 5 fraction when additional separately prepared cell-sap RNA is added. We found that even if rat-liver cell-sap RNA is added to guinea-pig mammary gland pH 5 fraction the amount of coupled amino acid is proportional to the

total cell-sap RNA concentration. The results of one such experiment are recorded in Fig. 2.

The experiments recorded in Figs. 3 and 4 were carried out to study the dependence of the extent of cell-sap RNA-amino acid combination on amino acid concentration. Unlabelled amino acids were added to make up the total concentration, and though DL-amino acids were used the concentrations of L-amino acids are given and the counts are divided by the specific activities of the amino acid preparations used. A separate experiment with glycine, extended to a concentration of 0.02M, showed that the increase in cell-sap RNA-glycine combination remained linear.

When the time course of cell-sap RNA-amino acid combination is followed one can always observe a reversal of this process after incubation for about 30 min. (see Fig. 1). We therefore incubated [14 C]amino acid-labelled, precipitated and washed pH 5 fraction, which had been redissolved in medium A, in the presence of 0.01M-ATP, as indicated above. Fig. 5A shows the results of such a delabelling experiment carried out with [14 C]-leucine-labelled pH 5 fraction. The reversibility of the removal of leucine was tested in a parallel experiment (Fig. 5B) in which [14 C]leucine was added 15 min. after the beginning of incubation in the amino acid-free medium. To determine whether the time course of the removal of leucine from its compound with pH 5 fraction was the same when the compound formation occurred at relatively high leucine concentration, pH 5 fraction, prelabelled with 0.01M-leucine, was subsequently incubated in an amino acid-free medium as above (Fig. 5C). This removal of amino acids from the compound was shown to be quite independent of the presence or absence of ATP, whereas there was the same requirement for ATP for the reversal of this process as for the initial compound formation.

Fig. 6 shows the results of experiments on the comparative effect of L-[14 C]leucine and DL-[14 C]-leucine on the labelling of pH 5 fraction. The total concentration of leucine was the same (80 μ M) in both experiments. The counts obtained from the samples of the experiment with DL-leucine were multiplied by 0.66/0.41 to correct for the greater specific activity of the L-leucine preparation.

Two experiments to investigate competitive inhibition of amino acids for their combination with pH 5 fraction are recorded in Fig. 7. As can be seen, there is some inhibition of leucine coupling in the presence of high concentrations of valine, whereas there is no inhibition of valine coupling in the presence of high concentrations of leucine. Similarly, we found that glycine (M) did not reduce the final amount of valine combined with pH 5 fraction, though it did slow down the rate of coupling process.

Two types of experiment were carried out to investigate whether the reactions of glutamic acid and glutamine with pH 5 fraction are independent of each other. DL-[14 C]Glutamic acid (0.25 μ C/ml.), ATP (0.01M) and pH 5 fraction were incubated for

Fig. 1. Combination of L-[14 C]leucine with total pH 5 fraction (O) and with the protein of pH 5 fraction (●). Incubation was as described in the text; [14 C]leucine 57 μ M, 0.5 μ C/ml.

Fig. 2. Combination of [14 C]glycine with guinea-pig mammary gland pH 5 fraction (●; 0.35 mg. of cell-sap RNA/ml.) and the effect of added (0.65 mg./ml.) rat liver cell-sap RNA (Δ) on glycine uptake by this fraction. Incubation mixture was as described in the text; [14 C]glycine 50 μ M, 0.25 μ C/ml. The broken line (O) is calculated for the expected uptake if this is proportional to increase in cell-sap RNA concentration.

Fig. 3

Fig. 4

Figs. 3, 4. Combination of several amino acids with pH 5 fraction over a range of amino acid concentration. Incubation was for 30 min. as described in the text.

Fig. 5. Removal of $[^{14}\text{C}]$ leucine from its compound with pH 5 fraction in amino acid-free medium: (A) labelled with L-leucine ($50\ \mu\text{M}$, $0.37\ \mu\text{Ci/ml.}$) for 30 min. before incubation; (B) $[^{14}\text{C}]$ leucine ($0.3\ \mu\text{Ci/ml.}$) added after 15 min.; (C) labelled with L-leucine ($10\ \text{mM}$, $1\ \mu\text{Ci/ml.}$) for 30 min. before incubation.

10 min. (1) without any addition, (2) in the presence of unlabelled L-glutamic acid (0.01M), (3) in the presence of unlabelled L-glutamine (0.01M) and (4) in the presence of Elliott's (1953) glutamine synthetase system. The counts/min./ cm.^2 obtained for samples of pH 5 fraction subsequently prepared from the above four incubation mixtures in the usual manner were 104, 0, 32 and 210 respectively. Though lack of stability of glutamine caused some dilution of glutamic acid label in (3), the relatively small dilution at such a large carrier concentration indicates separate specificity for glutamic acid and glutamine. The increase of labelling of pH 5 fraction in the presence of $[^{14}\text{C}]$ glutamine and $[^{14}\text{C}]$ glutamic acid in incubation (4) corroborates this idea.

DISCUSSION

The experiments described in this paper were designed to investigate quantitative aspects of the reactions of cell-sap RNA with amino acids, with the view of elucidating the particular functions of these reactions in the overall process of protein biosynthesis. The mechanism of the condensation of amino acids to peptide chains for the formation of protein molecules has to fulfil two tasks. First, it has to couple the endergonic peptide-bond forma-

tion with an exergonic reaction to make the overall reaction thermodynamically possible. Secondly, it has to include a step or series of steps which determine the specific sequence in which the amino acids condense. The first part of this problem has been solved in principle by the discovery of enzyme systems which catalyse the formation of the high-energy amino acid adenylates from amino acids and ATP. It is widely believed that specific amino acid-activating enzymes will be found for each individual amino acid; that it has not yet been shown whether there are 20 distinct activating enzymes or fewer enzyme systems capable of activating all 20 amino acids is probably due to the use of inadequate methods of fractionation and enzymic assay of the particle-free supernatant. As anticipated, preliminary studies of amino acid activation in mammary tissue (Gutfreund, 1958; Fraser & Gutfreund, 1958) gave results which have to be revised, and far more detailed investigations of these enzyme preparations and reactions are in progress in this Laboratory.

Since it was shown by Hoagland *et al.* (1958) that the reactions of amino acids with cell-sap RNA occur after the enzymic formation of amino acid

adenylates and before the incorporation of the amino acids into protein in the microsomal particles, one would like to find any possible role of cell-sap RNA in the determination of the specific sequence. It was reported by Fraser & Gutfreund (1958) that the relative number of specific places available on cell-sap RNA for different amino acids appears to bear some relation to their abundance in the protein synthesized. The more detailed experiments with six amino acids described in this paper confirm this point. For most of our cell-sap RNA-amino acid-coupling experiments we took as a measure of the quantities of [14 C]amino acids bound to cell-sap RNA the radioactivity of suitably prepared specimens of the pH 5 fraction, which contained protein as well as RNA. We have carried out a number of experiments to separate cell-sap RNA and protein of amino acid-labelled pH 5 fraction. Fig. 1 shows the relative labelling of cell-sap RNA and protein under a variety of conditions. It seems likely that the combination of protein with [14 C]amino acids is due to a non-specific reaction of activated amino acids, which is further discussed below. We have also shown that the addition of cell-sap RNA to pH 5 fraction increases the uptake of amino acids on incubation and that this uptake is proportional to total cell-sap RNA concentration. As shown in Fig. 2, there is a proportional increase in amino acid uptake when cell-sap RNA prepared from rat liver is added to

Fig. 6. Combination of DL-[14 C]leucine (●; 0.41 μ C/ml.) and L-[14 C]leucine (○; 0.66 μ C/ml.) with pH 5 fraction. Total leucine concentration in each case was 80 μ M.

Fig. 7. Inhibition of the combination of DL-leucine by DL-valine. Concentrations of [14 C]amino acids and unlabelled amino acids in the four incubation mixtures were as follows: ○, DL-[14 C]leucine, 98 μ M; ●, DL-[14 C]leucine, 98 μ M, and unlabelled DL-valine, 0.318 M; △, DL-[14 C]valine, 0.28 mM; ▲, DL-[14 C]valine, 0.28 mM, and unlabelled L-leucine, 0.15 M.

pH 5 fraction from guinea-pig mammary gland. It would, of course, be of great interest to have precise data from the number of nucleotides per binding site for each individual amino acid. The evaluation of such figures is complicated by the form of the saturation curves for cell-sap RNA-amino acid coupling. A first inspection of the plots of amino acid concentration against a measure of the bound amino acid residues (Figs. 3, 4) shows that the process is biphasic. First there is a rapid increase of cell-sap RNA-amino acid compound formation with increasing concentration of amino acids up to about $20\ \mu\text{M}$, and then there follows a slower continuous increase in compound formation proportional to amino acid concentration. One can calculate from our data for some amino acids at high concentrations (about 0.1M) that they combine to the extent of one amino acid residue per nucleotide. It appears likely that the cell-sap RNA-amino acid combination at low amino acid concentration involves occupation of sites specific for each amino acid, whereas the extensive further combination involves the non-specific reaction of activated amino acid adenylates with hydroxyl and other groups of cell-sap RNA. That such non-specific reactions occur has been demonstrated by Castelfranco, Moldave & Meister (1958).

Two different experiments were carried out to investigate the properties of cell-sap RNA-amino acid compounds formed at relatively high amino acid concentration in an attempt to distinguish the linkages thus formed from those which are thought to be on the specific sites. We found that incubation of the total pH 5 fraction with ATP and α -glycine or 0.15M -leucine had no significant effect on the combination of cell-sap RNA with valine. It appears therefore that the specific places for individual amino acids are still available even if very extensive combination with what are assumed to be non-specific sites has taken place. Another property of extensively coupled cell-sap RNA is that the amino acids are removed from it under the same conditions and at the same rate as from the specific cell-sap RNA-amino acid compounds. We have studied the time course and conditions of this 'delabelling' process, and though we have little information about the chemical mechanism involved, our data can be correlated with chemical investigations carried out in other laboratories. Fig. 5 shows that if [^{14}C]leucine-labelled pH 5 fraction precipitated at pH 4.9 is resuspended in medium A and 0.01M -ATP, leucine is rapidly removed from the compound. It has been shown that this process is reversible since on further addition of leucine after 15 min. the compound is reformed (Fig. 5B). Preliminary experiments indicate that at least some of the leucine liberated is associated with material absorbing at $260\text{ m}\mu$.

In view of the experiments reported by Hecht, Stephenson & Zamecnik (1958) and by Zachau, Acs & Lipmann (1958), it seems to us reasonable to suggest that the enzymic labelling of cell-sap RNA is reversed by enzymic removal of an amino acid-adenosine monophosphate compound. When there is an ample supply of free amino acids and ATP in solution the rate of compound formation is considerably greater than the reverse process. This reversal also occurred in the absence of ATP and is probably due to ribonuclease. A reversal of cell-sap RNA-amino acid compound formation, which is probably of a different nature, was reported by Glassman, Allen & Schweet (1958), who found that if the compound is incubated with activating enzyme, [^{14}C]adenosine monophosphate and pyrophosphate in the absence of free amino acids and ATP, then free amino acids and [^{14}C]ATP are produced.

It has been mentioned above that incubation of pH 5 fraction with glycine or leucine does not interfere with cell-sap RNA-valine compound formation. We have been able to show, however, that some competition between amino acids can occur. Large concentrations of valine, for instance, will interfere with the cell-sap RNA-leucine combination (Fig. 7). One can speculate here that of the two structurally similar amino acids, leucine and valine, the smaller one will fit into the site of the larger one but not vice versa.

As indicated in the description of individual experiments, data for the quantitative aspects of the specificity of cell-sap RNA-amino acid combination were obtained by the use of DL-[^{14}C]amino acids. Synthetic DL-amino acids are much less likely to be contaminated with other amino acids than the natural L forms, which are prepared chromatographically. A comparison of the cell-sap RNA-leucine combination during incubation of pH 5 fraction, ATP and L-[^{14}C]leucine or DL-[^{14}C]leucine shows a very interesting difference. In Fig. 6 the time course of the reaction is shown for the two forms of [^{14}C]leucine; the total leucine concentration and the specific radioactivity are the same for both curves. It is likely that only the L-isomer is coupled to cell-sap RNA, and inspection of the saturation curves (Fig. 4) shows that the effect of half the concentration of L-isomer in DL-leucine should be a reduction of compound formation by about only 10%, whereas it can be seen in Fig. 6 that on incubation with DL-leucine only 60% of the compound formation occurs as compared with the incubation with L-leucine. That this effect is not just a slowing down through competitive inhibition by the D-isomer of an enzyme system is indicated by the fact that the ratio of compound formation for the two forms of leucine remains the same over the whole time course of the experiment.

It was suggested to us by Dr R. B. Loftfield that the most likely explanation for the excess of labelling in the presence of L-[^{14}C]leucine is the presence of small quantities of other amino acids. This point does, however, need further examination.

The importance of cell-sap RNA in the specific selection of amino acids before their incorporation into the peptide-chain sequence is further emphasized by our experiments with glutamic acid and glutamine. It has been shown by Sansom & Barry (1958) that asparagine and glutamine are incorporated into milk protein independently from aspartic acid and glutamic acid respectively. We were able to show that the binding sites on cell-sap RNA can distinguish between glutamine and glutamic acid. Though the instability of glutamine made it impossible to obtain quantitative information for this amino acid it seems likely that the cell-sap RNA-coupling process is the point in protein synthesis at which the distinction occurs between the dicarboxylic amino acids and their amides.

SUMMARY

1. Systems were developed for the study of the reactions of the enzyme systems and cell-sap ribonucleic acid of guinea-pig mammary tissues with [^{14}C]amino acids and adenosine triphosphate.

2. It was shown that the quantities of amino acids taken up by such systems depend on the amino acid concentration and on the cell-sap ribonucleic acid concentration.

3. Though it is clear that there are specific sites for each amino acid studied, the precise number of sites is difficult to evaluate because of non-specific reactions.

4. From the data obtained the conclusion can be

drawn that the extent of combination of amino acids with cell-sap ribonucleic acid determines the quantities of each amino acid to be incorporated into protein. This step may also be responsible for conferring additional specificity on amino acids to determine their addition to polypeptide chains in the correct position in the sequence.

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Turnover of Nucleic Acids in a Non-Multiplying Animal Cell

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Turnover of nucleic acids may be defined as the continuous renewal of these compounds within the cell by a balanced process of degradation and resynthesis. The idea that ribonucleic acids turn over in this way is derived from experiments on animals (Smellie, 1955). But the complexity of animal experiments does not lend itself to measurement of the rate of intracellular turnover (Cohn, 1957), and the results which have been obtained in such experiments are difficult to interpret. Animal

experiments have failed to decide whether turnover of the deoxyribonucleic acids occurs at all. There appear to have been only two studies of nucleic acid turnover in animal cells cultivated *in vitro*. In the first of these (Siminovitch & Graham, 1956) cells in exponential growth were studied. The retention of radioactive phosphorus in the nucleic acids was used to measure turnover, but no turnover was detected either in ribonucleic acid or in deoxyribonucleic acid. In the second study