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## *LIPOAMINO ACIDS AS PRECURSORS IN THE SYNTHESIS OF AUTHENTIC PROTEINS BY HOMOGENATES OF HEN OVIDUCT*

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It has been a general observation that when a cell is destroyed by homogenization, severe changes result in the ability of the tissue to synthesize proteins. In quantitative terms, a loss of 95–100 per cent in the ability to incorporate amino acids is most common.[1] Qualitatively, the usual observation has been that all or nearly all of the incorporated amino acids are in noncharacterizable products rather than in the normal proteins synthesized by the tissue. In some cases, recognizable proteins have been clearly identified but even in these cases, a considerable amount of the noncharacterized incorporation has taken place.[2–4] Early studies with rat liver showed that the more efficient technique of cell homogenization destroyed more of the tissue's ability to synthesize proteins.[5] Recent investigations with *E. coli* showed that the manner of cell breakage influenced structural associations of ribosomes and membranes.[6–8] More recent studies with microbial systems provide strong evidence that acellular systems are capable of authentic protein synthesis.[9, 10]

Although the major emphasis in studies on protein synthesis has centered on ribosomes as the structural requirement, a considerable amount of published evidence has pointed to cell membranes as also playing a key role.[1, 11] Similarly, the importance of nucleic acids in the process has occupied a central position in most major investigations. Several laboratories have reported on the occurrence of lipoamino acids.[12] This is a generic term to describe a group of compounds and complexes which are formed when amino acids are added to a variety of different tissues. Although chemical structures have been assigned to some lipoamino acids, most of them are at the moment chemically uncharacterized. The kinetic behavior with respect to uptake and loss of radioactive amino acids for some of these materials has suggested the possibility of their importance in protein synthesis but such a role, if any, has remained unclear.

The current work offers a basis for clarification of the unresolved issues discussed in this introduction.

*Methods.—General description:* The incubations were carried out in two phases. The first phase was designed to yield lipoamino acids from intact cell incubations. In the second phase the radioactive lipoamino acid, in an ultrasonically produced water emulsion, was incubated in a homogenate to produce radioactive proteins. Finally, the radioactive proteins were shown to be authentic hen oviduct proteins. Details of these procedures follow.

*Formation and isolation of lipoamino acids:* U-$C^{14}$ alanine (0.67 mC, specific activity 36.6 mC/mmole) in 5 ml of medium was incubated with 5 gm of hen oviduct mince at 37° for 15 min as previously described.[13] After incubation, the supernatant fluid was removed by centrifugation and immediately used again for a second incubation with 5 gm of fresh tissue. Each labeled tissue preparation was extracted separately. The tissue was collected by centrifugation, washed twice with fresh buffer, and then blended with 10 ml of alcohol that had been previously bubbled with $N_2$. Blending was performed with a VirTis "23" homogenizer for 3 min with the tissue suspension in a glass vessel immersed in ice water. After the homogenate had been transferred to another vessel, the homogenizer was rinsed with 10 ml of fresh alcohol and this was added to the homogenate. One ml of concentrated HCl was mixed with the 20 ml of homogenate and the suspension was allowed to stand 15 min at room temperature. The supernatant fluid was removed by centrifugation and the tissue was washed successively once with alcohol, three times with 3:1 alcohol:ether at 60–65° for 5 min each time, and once with absolute ether. All extracts were combined and the solvents removed *in vacuo* below 40°. The resulting oil was extracted successively with small volumes of $CHCl_3$ and 9:1 $CHCl_3$:$CH_3OH$ which were pooled and filtered through Whatman #1 paper. The initially cloudy filtrate was cleared by further filtration. The solution was concentrated and chromatographed on a 5-gm column of silicic acid.[14] The column was eluted successively with 25-ml portions of $CHCl_3$, 9:1 $CHCl_3$:$CH_3OH$, 4:1 $CHCl_3$:$CH_3OH$, 1:1 $CHCl_3$:$CH_3OH$, and absolute $CH_3OH$. Table 1 shows recoveries of radioactivity in each fraction.

U-$C^{14}$ glycine (0.46 mC, specific activity 24.4 mC/mmole) was employed in exactly the same manner as described above for U-$C^{14}$ alanine to yield two sets of silicic acid column fractions. Recoveries of radioactivity are listed in Table 1.

*Preparation of lipoamino acid-water dispersions:* A solution of lipoamino acid in the organic solvent with which it was eluted from the silicic acid column was concentrated below 40° under a stream of $N_2$ and transferred to a liquid scintillation counting vial in a small volume of solvent. The solvent was thoroughly removed by evaporation under $N_2$ and 4–6 ml of water added to the oil. The vial was set in an ice-water bath and a stream of $N_2$ was played on the surface of the liquid. Ultrasonic treatment at 20,000 cps was performed with a "Branson" S-75 sonifier at a power setting of 6 for two or three 1-min periods. An almost water-clear emulsion was made for each lipid fraction except the lipid eluted with $CHCl_3$, which remained milky after treatment. A one-tenth volume of 10-fold concentrated standard buffer salts–glucose solution was added. The emulsions were kept on ice and used for homogenate incubations.

*Incubation of lipoamino acid emulsions with tissue homogenates:* Twenty-five grams of oviduct tissue was forced through a tissue press to remove connective tissue. The tissue was then ho-

TABLE 1

INCORPORATION OF AMINO ACIDS INTO LIPID FRACTIONS OF HEN OVIDUCT MINCE

| | Alanine Experiment | | Glycine Experiment | |
|---|---|---|---|---|
| | Incubation 1 | Incubation 2 | Incubation 1 | Incubation 2 |
| Total radioactivity in $CHCl_3$:$CH_3OH$ extract | 7.8* | 3.4 | 2.7 | 2.9 |
| Radioactivity applied to silicic acid column | 7.0 | 3.1 | 2.7 | 2.9 |
| Radioactivity in column fractions | | | | |
| $CHCl_3$ | 0.58 | 0.18 | 0.15 | 0.16 |
| 9:1 $CHCl_3$:$CH_3OH$ | 1.2 | 0.47 | 0.62 | 0.62 |
| 4:1 $CHCl_3$:$CH_3OH$ | 2.4 | 0.54 | 0.27 | 0.86 |
| 1:1 $CHCl_3$:$CH_3OH$ | 1.4 | 0.65 | 0.48 | 1.26 |
| $CH_3OH$ | 0.04 | 0.02 | 0.20 | 0.02 |
| Total eluted | 5.62 | 1.86 | 1.72 | 2.92 |
| % Recovery | 80 | 60 | 64 | 100 |

* Counts per minutes in millions, as determined in a Nuclear-Chicago model 725 liquid scintillation spectrometer at 80% efficiency.

mogenized for two periods of 90 sec with 25 ml of buffer in a Potter-Elvehjem glass homogenizer with a Teflon pestle, immersed in ice. With a pipette having a wide opening, 2 ml (1 gm tissue) of the suspension was pipetted into centrifuge tubes. The suspension was centrifuged in a clinical centrifuge for about 3 min and the supernatant fluid removed. The supernatant fluid was replaced either with 1 ml of $C^{14}$ sonicated lipoamino acid emulsion or free $C^{14}$ amino acid in buffer. The suspensions were incubated for 90 min at 37°. In the glycine experiments, 0.8 gm tissue and 0.8 ml supernatant fluid were used in each incubation. In the alanine experiments, all of the proteins were precipitated with 10% TCA and prepared for counting.[13] In the glycine experiments, the supernatant fluid was removed by centrifugation in a clinical centrifuge for 3 min. The sedimented proteins were treated with 10% TCA and prepared for counting. Ovalbumin was isolated from the supernatant by electrophoresis on paper.[15] The area containing ovalbumin was cut out and eluted with 1% sodium sulfate solution. The solution was adjusted to pH 4.5 with 1 *M* acetic acid and the ovalbumin was coagulated by boiling for 5 min. The protein was washed successively with distilled water, alcohol, and ether.

*Identification of labeled proteins from homogenate:lipoamino acid incubations with those from intact cell:free amino acid incubations:* (a) *Formation of peptide digest by treatment with pepsin:* Fifty mg of dry protein was treated with 0.25 ml 98–100% HCOOH. (The treatment of the insoluble proteins with concentrated formic acid to facilitate pepsin digestion was suggested by Dr. C. B. Anfinsen, Jr.) First, 4.75 ml of water was added and then 0.5 mg of pepsin in 0.05 ml of 0.01 *N* HCl. For different amounts of protein, the same proportions were maintained. The suspension was shaken at room temperature. Digestion was followed by assay of increase of ninhydrin-positive material and found to be complete in 2 hr in all cases. Solvents were removed quickly *in vacuo* below 60°. The residue was extracted with small aliquots of 0.1 *M* NaCl. This resulted in all cases in a concentrated peptide extract of 0.5–1 ml and an insoluble residue.

(b) *Column chromatography:* In the experiment of Figure 1 the pepsin-solubilized peptides from 50 mg of total protein labeled with $C^{14}$ alanine in a volume of 1 ml 0.1 *M* NaCl were placed on a 25-ml column (13 × 200 mm) of DEAE Sephadex A-25 that had been previously treated with KOH and washed extensively with water. The column was developed with a linear gradient of NaCl from 0 to 0.5 *M* concentration for fifty 1-ml fractions. Afterwards, four 10-ml fractions were collected with 0.5 *M* NaCl. Pepsin treatment solubilized 66% of the radioactivity from the mince-incubated proteins and 62% from the homogenate incubation. Recovery of applied radioactivity was quantitative in both cases. The fractions were assayed for biuret-positive[16] and ninhydrin-positive materials and radioactivity as described below for the experiment of Figure 2.

In Figure 2 the pepsin-solubilized peptides from 50 mg of sedimented protein-labeled with glycine in a volume of 1 ml was placed on a 25-ml (13 × 210 mm) column of Sephadex G-25 that had been equilibrated with a solution of 0.1 *M* NaCl. Fractions of 1 ml were collected, and 50-$\mu$l aliquots were withdrawn for biuret[16] and ninhydrin determinations and the remainder was counted in Bray's solution[17] in a liquid scintillation counter. Pepsin solubilized 90% of the radioactivity from the mince-labeled proteins and 70% from the homogenate incubation. In the pattern shown in Figure 2 for the mince, 70% of the applied radioactivity is accounted for and 60% in that for the homogenate.

In Figure 3 the pepsin-solubilized peptides from 20 mg of electrophoretically pure ovalbumin in a volume of 0.5 ml were applied to a 10-ml column (11 × 140 mm) of DEAE Sephadex A-25. The column was developed with a linear gradient of NaCl 0–1 *M*, and 1.3-ml fractions were collected. A 70-$\mu$l aliquot was taken for ninhydrin assay and 80-$\mu$l for biuret.[16] The remainder was assayed for radioactivity in Bray's solution in a liquid scintillation counter. Pepsin treatment solubilized approximately 70% of the total radioactivity from both the mince and homogenate-incubated proteins. Recovery of radioactivity from the columns was quantitative.

*Results and Discussion.*—(1) *Incorporation of lipoamino acids into the proteins of homogenates:* Table 2 shows the incorporation of various $C^{14}$ lipoalanines and $C^{14}$ free alanine into the proteins of intact cell preparations (mince) and into broken cell preparations (homogenate). The lipoamino acids were incorporated less efficiently into the intact cell preparation than was the free amino acid. Prior to the homogenate incubations, the soluble part of the cell content was discarded and replaced with fresh medium containing either $C^{14}$ alanine or $C^{14}$ lipoalanine. Therefore, the specific activity of the free $C^{14}$ alanine pool was much higher for homogenate incubations than for the mince. Because of this, relative incorporating ability for $C^{14}$ alanine in the homogenate compared to the mince would be much less than the 2.5 per cent indicated in the table (i.e., compare 0.2 to 7.6). The relative inability of the homogenate to incorporate free amino acids has been studied previously.[18] This is a general characteristic of most tissue systems studied.[1] The lipoamino acids were all more efficiently incorporated into protein than was the free amino acid. The 9:1 fraction of lipoalanine was by far the most active and at least ten times more efficiently incorporated than the free amino acid. The specific activity of the $C^{14}$ alanine introduced in this experiment was much higher than that of $C^{14}$ lipoalanine since lipoalanine was diluted by the $C^{12}$ pools present in the initial incubations from which the $C^{14}$ lipoalanines were isolated. Therefore, the relatively greater incorporation rate for $C^{14}$ lipoalanine may represent a minimum level.

TABLE 2

INCORPORATION OF $C^{14}$ ALANINE AND $C^{14}$ LIPOALANINE INTO PROTEINS OF MINCE AND HOMOGENATES

| Tissue | Radioactive precursor* | Radioactivity in incubation† | Radioactivity Incorporated into Proteins | |
|---|---|---|---|---|
| | | | Observed cpm‡ | % of added radioactivity§ |
| Mince | Alanine | 225,000 | 200 | 7.6 |
| Mince | Mixed lipoalanines | 225,000 | 120 | 4.6 |
| Homogenate | $CHCl_3$-lipoalanine | 215,000 | 8 | 0.25 |
| Homogenate | 9:1-Lipoalanine | 235,000 | 54 | 1.6 |
| Homogenate | 4:1-Lipoalanine | 900,000 | 110 | 0.7 |
| Homogenate | 1:1-Lipoalanine | 500,000 | 18 | 0.2 |
| Homogenate | $CH_3OH$-lipoalanine | 14,000 | 2 | — |
| Homogenate | Mixed lipoalanines | 225,000 | 20 | 0.6 |
| Homogenate | Nonchromatographed lipoalanines | 390,000 | 37 | 0.75 |
| Homogenate | Alanine | 250,000 | 6.4 | 0.2 |
| Homogenate | Alanine | 500,000 | 12.7 | 0.2 |
| Homogenate | Alanine | 1,000,000 | 21 | 0.15 |

* The designations $CHCl_3$, 9:1, 4:1, 1:1, $CH_3OH$ refer to the silicic acid column fractions obtained by chromatography of the amino acid–labeled lipids extracted from a preliminary incubation with the mince. "Mixed lipoalanines" were obtained by mixing together portions of all of the eluted fractions. The observed incorporation for "Mixed" and "Nonchromatographed" total lipids were close to the sums calculated for each component to be acting independently.

† Counts per minute determined in a Nuclear-Chicago model 725 liquid scintillation spectrometer at 80% efficiency.

‡ Counted in a solid state at "infinite thickness" with an end-window counter. Efficiency was 1.4 to 1.8% depending on actual weight of sample. All samples were done in duplicate and counted for at least a total of 1,500 counts each in a Nuclear-Chicago model C-110B gas-flow counter, having a background of less than 4 cpm.

§ Calculated after correcting for efficiencies of radioactivity assays.

TABLE 3

INCORPORATION OF $C^{14}$ GLYCINE AND 9:1 FRACTION $C^{14}$ LIPOGLYCINE INTO SEDIMENTABLE PROTEINS OF MINCE AND HOMOGENATE*

| Tissue | Radioactive precursor† | Time of incubations in min | Additions | Radioactivity Incorporated into Proteins: Observed cpm‡ | Radioactivity Incorporated into Proteins: % of added radioactivity§ |
|---|---|---|---|---|---|
| Mince | Glycine | 0 | — | 1.5 | 0.04 |
| Mince | Glycine | 90 | — | 64 | 1.8 |
| Homogenate | Lipoglycine | 0 | — | 52** | 1.4 |
| Homogenate | Lipoglycine | 5 | — | 77 | 2.1 |
| Homogenate | Lipoglycine | 45 | — | 86 | 2.4 |
| Homogenate | Lipoglycine | 90 | — | 117 | 3.0 |
| Homogenate | Lipoglycine | 90 | — | 99 | 2.7 |
| Homogenate | Lipoglycine | 90 | 5 Mg $C^{12}$ glycine | 99 | 2.7 |
| Homogenate | Lipoglycine | 90 | $C^{14}$ Glycine to double concentration | 130 | 3.5 |
| Homogenate | Glycine | 0 | — | 5 | 0.13 |
| Homogenate | Glycine | 90 | — | 11 | 0.32 |
| Homogenate | Glycine | 90 | 5 Mg $C^{12}$ glycine | 7 | 0.15 |

* All flasks contained 130,000 cpm except flask no. 9 which contained 260,000 cpm as determined in a Nuclear-Chicago model 725 liquid scintillation spectrometer at 80% efficiency.
† The lipoglycines were all from the 9:1, $CHCl_3$:$CH_3OH$ fraction eluted from silicic acid column chromatography of lipids extracted from a mince after incubation with $C^{14}$ glycine.
‡ Counted in a solid state at near "infinite thickness" with an end-window counter. Efficiency was 2.1–2.7%, depending on actual weight of sample. All samples were counted for at least a total of 1,280 counts in a Nuclear-Chicago model C-110B gas-flow counter having a background of 5 cpm.
§ Calculated after correcting for efficiencies of radioactivity assays.
** See text for discussion of 0 time value.

The experiment shown in Table 3 was performed with $C^{14}$ glycine and the $C^{14}$ lipoglycine contained in the 9:1 $CHCl_3$:$CH_3OH$ eluate from silicic acid column chromatography. The same comments concerning relative specific activities for the alanine pools as discussed above would apply to the glycine pools in this experiment. The zero time points for the homogenates were not true zero times since cell homogenate samples were first centrifuged to separate out sedimentable proteins. These operations involved approximately 2–3 min time. Therefore, the level of incorporation for the lipoglycine at zero time would consist of rapidly incorporated precursor plus any adsorbed material. If the unlikely assumption were made that the "zero time" value was due entirely to adsorbed material, the increment of incorporation observed during the incubation is still impressive. However, as is shown below, virtually all of the radioactivity incorporated in the lipoglycine-homogenate incubation corresponded to that incorporated in the glycine-mince incubation. This was revealed by the similarity of the peptides released from the radioactive proteins by pepsin treatment. Since the "zero time" value was approximately 50 per cent of that at 90 min, such a comparison for the proteins labeled in the homogenate and mince incubations would have shown appreciable dissimilarity if the fast labeled material was due to spurious incorporation. Therefore, the "zero time" value represents mostly authentically incorporated radioactive amino acid. The addition of 5 mg of $C^{12}$ glycine was sufficient to abolish the small level of incorporation observed above the zero time value when $C^{14}$ glycine was the precursor. When $C^{14}$ lipoglycine was the precursor, little or no diminution occurred in the extent of incorporation. The addition of $C^{14}$ glycine with $C^{14}$ lipoglycine resulted in a comparative 3.5 per cent level of incorporation. The higher of the two values obtained with $C^{14}$ lipoglycine alone was 3.0 per cent and the level obtained with $C^{14}$ glycine alone was 0.32 per cent. Therefore, the observed level of 3.5 per cent represents a value close to the additive levels. This means that the simple presence of the 9:1 lipid fraction cannot markedly enhance the incorporation of free $C^{14}$ glycine

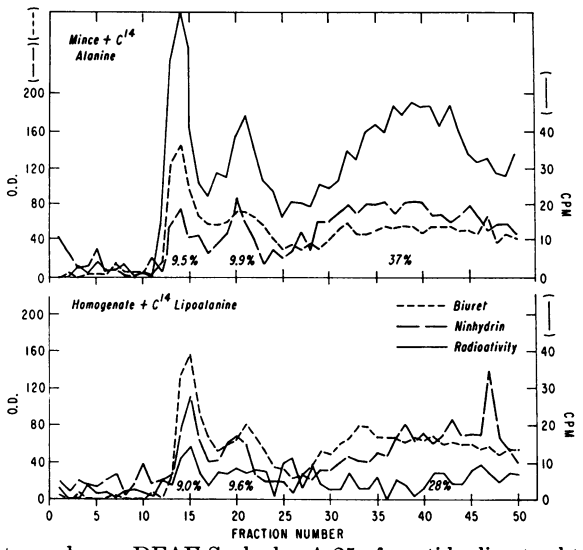


FIG. 1.—Chromatography on DEAE Sephadex A-25 of peptide digests obtained by treatment of the total proteins of hen oviduct with pepsin. The column was developed with a linear gradient of NaCl from 0 to 0.5 *M*. The upper figure shows results from an incubation of the intact mince with $C^{14}$ alanine. The lower figure shows results from an incubation of homogenate with $C^{14}$ lipoalanine from the 9:1 fraction of silicic acid column chromatography. An incubation of homogenate plus $C^{14}$ alanine yielded too low an incorporation for any characterization. Flow rates were 10 ml. per 5.43 min and 10 ml per 5.25 min for the upper and lower figures, respectively. Details are given in the text.

into protein and that the improved status of lipoglycine as a precursor for protein synthesis was a result of the prior incubation in which it was formed.

(2) *The authenticity of proteins synthesized in homogenates from $C^{14}$ lipoamino acids:* (a) *Total proteins labeled with alanine:* The total proteins obtained in the experiment shown in Table 2 after washing with cold trichloroacetic acid, alcohol, ether, and hot trichloroacetic acid were digested with pepsin and chromatographed on DEAE Sephadex A-25. When free $C^{14}$ alanine was digested along with the proteins and subsequently chromatographed under similar conditions, a single radioactive peak was obtained with a maximum around fraction no. 26. Figure 1 shows that the distribution of radioactivity among the peptides obtained by pepsin hydrolysis is very similar for the two samples. The locations of the major peaks and troughs coincide. The first and second peaks contained 9.5 and 9.9 per cent, respectively, of the applied radioactivity in the case of the mince and 9.0 and 9.6 per cent, respectively, in the case of the homogenate. The region from fractions 27 to 50 contained too little radioactivity in the case of the homogenate to know if a close correspondence exists. When 0.5 *M* NaCl was added after the gradient and 10-fractions were collected, additional radioactivity was recovered from the columns. Fifty-seven per cent of the applied radioactivity was recovered in the 50 fractions shown in Figure 1 from the mince incubation and 50 per cent from the homogenate incubation. Subsequent elution with 0.5 *M* NaCl removed 25, 8, 5, and $< 1$ per cent in four fractions from the column with the mince hydrolysate and 16, 16, 16, and 4 per cent from the column with the homogenate hydrolysate. Recovery of applied radioactivity was complete.

(b) *Sedimentable proteins labeled with glycine:* The sedimentable proteins from

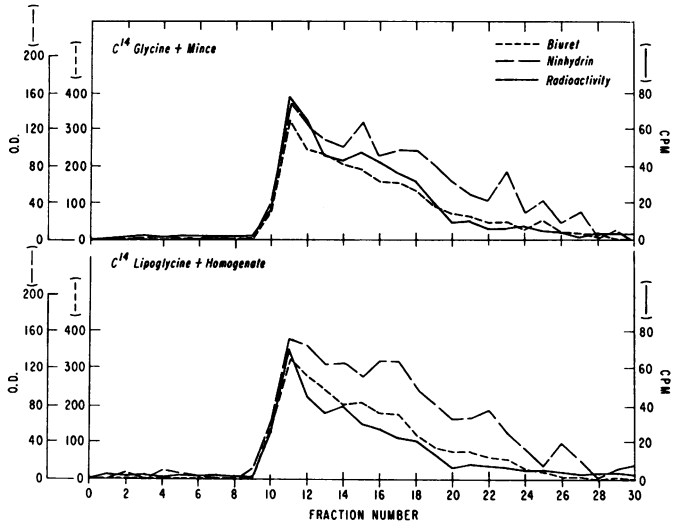


FIG. 2.—Chromatography on Sephadex G-25 of peptide digests obtained by treatment of the sedimented proteins of hen oviduct with pepsin. The column was developed with 0.1 *M* NaCl. The upper figure shows results from an incubation of intact mince with $C^{14}$ glycine. The lower figure shows results from an incubation of homogenate with $C^{14}$ lipoglycine from the 9:1 fraction of silicic acid column chromatography. An incubation of homogenate with $C^{14}$ glycine yielded too low an incorporation for any characterization. Flow rates were 10 ml per 14 min and 10 ml per 13 min for the upper and lower figures, respectively. Details are given in the text.

the experiment shown in Table 3 were thoroughly washed with trichloroacetic acid and alcohol:ether, hydrolyzed with pepsin, and chromatographed on Sephadex G-25. Figure 2 shows that every peak, trough, and shoulder in the radioactivity elution pattern corresponds in the two cases. A similar digestion and chromatography of these proteins with free amino acids resulted in a single radioactive peak with a maximum in fraction no. 23.

(c) *Electrophoretically pure ovalbumin labeled with glycine:* Ovalbumin was isolated by electrophoresis on paper as described by Sanger and Hocquard.[15] The protein was hydrolyzed with pepsin and chromatographed on DEAE Sephadex A-25. Figure 3 shows the total correspondence for the peptide mixtures obtained from the

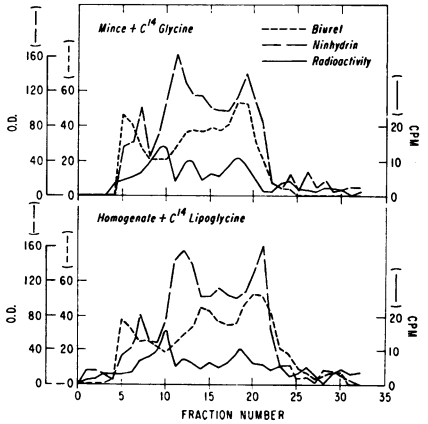


FIG. 3.—Chromatography on DEAE Sephadex A-25 of peptide digests obtained by treatment of electrophoretically pure ovalbumins of hen oviduct with pepsin. The column was developed with a linear gradient of NaCl from 0 to 1 *M*. The upper figure shows results from an incubation of intact mince with $C^{14}$ glycine. The lower figure shows results from an incubation of homogenate with $C^{14}$ lipoglycine from the 9:1 fraction of silicic acid column chromatography. An incubation of homogenate with $C^{14}$ glycine yielded too low an incorporation for any characterization. Flow rates were 10 ml per 3.83 min in both cases. Details are given in the text.

pure proteins synthesized in the mince from $C^{14}$ glycine and in the homogenate from $C^{14}$ lipoglycine. The two proteins appear to be identically labeled.

*Conclusion.*—The foregoing experiments have shown that lipoalanine and lipoglycine are very efficient precursors for the biosynthesis of the normal characteristic proteins of the hen oviduct under conditions where the free amino acids are very poor precursors. From the patterns of peptide labeling it seems that entire molecules are synthesized *de novo* in the homogenate incubations from these lipoamino acid precursors. Other studies involving a peptide mapping technique have shown that entire molecules of *de novo* synthesized proteins are absent from the lipoamino acid fractions used in this work.[19] These observations are in marked contrast to the poor incorporations obtained when free amino acids are used in broken cell preparations. A further important facet of the comparisons of free amino acids with lipoamino acids as direct precursors for protein synthesis in broken cell preparation is the fact that in most systems, with a few notable exceptions,[2-4, 9, 10] little or none of this total radioactivity incorporated is in the form of the normal proteins characteristic of the tissue. The present studies are consistent with various reports in the literature which have pointed to cellular membranes as the important site for cellular protein synthesis (as reviewed in refs. 1 and 11) and offer an experimental basis for coordinating acelullar and cellular studies on protein synthesis.

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