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METABOLIC PROPERTIES OF "LIPOPEPTIDES" ISOLATED FROM HEN OVIDUCT

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SUMMARY

Amino acids were found to rapidly enter and leave a purified lipopeptide material isolated from hen oviduct. It appears that amino acids leaving this material enter another purified lipopeptide. The labeling of the second material is inhibited by dinitrophenol, but not that of the first. The equilibration of these materials with the internal and external amino acid pools is considered as well as the interpretation of these findings in relation to the early stages of protein synthesis.

INTRODUCTION

In the preceding report, the isolation of lipopeptide materials is described[1]. This paper presents the results of experiments which demonstrate that amino acids are very rapidly incorporated into these compounds and that the amino acids in these substances are in a dynamic state. Evidence is also presented which suggests that amino acids from one of these lipopeptides flow into the other and that this transfer of radioactive amino acids is dependent upon reactions which are inhibited by dinitrophenol. Finally, observations are presented which indicate that a rapidly synthesized sugar moiety forms a part of the lipopeptide materials under study. The rapid amino acid passage through these materials is consistent with the idea that important phases of the early stages of protein synthesis may occur in association with membrane structures of the cells[2].

MATERIALS AND METHODS

The procedures employed for the isolation of these materials, as well as a description of the radioactive amino acids used, have already been published[1,3]. In the current studies, comparatively large quantities of tissue were used and consequently greater volumes of aqueous alcohol extracts had to be handled. In order to minimize the possibility of hydrolysis during the stages of concentrating these lipid extracts, the following modification of the previously published procedure was employed. The alcohol–water extract was kept separate from the alcohol and ether extract. The aq. alcohol extract

Abbreviation: PPO, 2,5-diphenyloxazole.

was frozen to a semi-solid state and carefully lyophilized. The chloroform extract of this fraction was later mixed with the chloroform extract of the alcohol–ether fraction for the further purification. Additional details as employed in this study are described below.

Procedures for obtaining the external and internal media

At the end of the incubation, the reaction mixture was centrifuged and the sedimented mince was washed two times with cold unlabelled buffer. These combined supernatant phases were brought to a concentration of 10 % trichloroacetic acid by the addition of a solution of 100 % (w/v) trichloroacetic acid. The precipitated proteins were further washed with cold trichloroacetic acid in the same manner as was done with the proteins obtained from the homogenate of the mince. The combined acid supernatants obtained from the buffer washes of the intact mince were the source of the external pools.

The washed tissue mince was homogenized and extracted with trichloroacetic acid as previously described[6]. The combined acid washes from the homogenized mince were the source of the internal pools.

Trichloroacetic acid was removed in an autoclave[6]. Salts were removed with the aid of a Dowex-50 column. The sample was passed through a column in the acid form. Amino acids and other cations were held, but anions were not retained. The amino acids were then eluted with 1 M NH_4OH, whereas the metallic cations remained adsorbed to the column.

Location of radioactive material on paper chromatograms

A 3-in strip was cut from the chromatogram from 0.5 in below the origin to just beyond the farthest point reached by the solvent. The strip was cut into 1.5-in sections to yield a series of 1.5 × 3-in rectangles. These rectangles, numbered sequentially from the origin, were rolled into cylinders which fit exactly to the interior surface of glass scintillation counting vials. A solution of 0.4 % PPO in toluene was added to just cover the paper (about 19 ml) and the vials were assayed for radioactivity in a Packard Tri-Carb Scintillation spectrometer.

Elution of radioactive material from the paper and preparation for chromatography on Dowex-2

The area of the paper which contained the radioactive material was cut out and eluted with 5 % formic acid in absolute ethanol. The alcohol–formic acid solvent was removed *in vacuo* below 40°. Small portions of absolute alcohol were added and the vacuum distillation repeated three times to remove formic acid. The oily residue was taken up in about 10 ml of 50 % alcohol and conc. NH_4OH was added to produce a pH reaction of 10 on pHydrion paper (Micro Essential Laboratory, Brooklyn, N. Y.). The solution was heated to 65–70° for 15 min to hydrolyze any easily hydrolyzable bonds. The solution was then concentrated by raising the temperature to about 80° and playing a jet of nitrogen on the surface. The oily residue which resulted was transferred with a small amount of alcohol to a Dowex-2 column and an equal amount of water was added to bring the concentration to 50 %. For some preparations, it was necessary to dissolve the oil in ether in order to apply it to the column. Under these circumstances, a stream of nitrogen was used to evaporate the ether from above the

column before the material was washed into the column with 50 % alcohol. The column was developed and fractions were assayed as previously described[1]. Radioactivity was followed by drying aliquots on ribbed 1.125-in metal planchets and counting in a Nuclear-Chicago Geiger flow-gas counter with a thin Micromil window.

Analysis of Dowex-2 fractions by high-voltage electrophoresis

Aliquots of the fractions were taken either directly or after hydrolysis and removal of fatty acid as described previously[1]. The residue from the aqueous fraction was electrophoresed at 5000 V in 3 % formic acid on Whatman 3-MM paper. A 3-in strip of the electrophoresis paper was cut into 1.5-in segments and analyzed for radioactivity in the manner described above for paper chromatograms.

Analysis of the paper strips for quantities of amino acids

After determination of radioactivity, the paper strips were removed from the scintillation vials and washed by soaking sequentially in two baths of chloroform. This process removed the toluene and PPO, but left the amino acids. A series of amino acids were applied to electrophoresis paper in known quantities and were similarly treated with PPO in toluene solution and then chloroform to serve as standards for the determination of the quantity of the amino acid on the paper. The paper strips were then treated with cadmium ninhydrin solution, according to the procedure of ATFIELD AND MORRIS[4]. The papers were then carefully folded into test tubes and eluted by letting stand with absolute methanol (usually 6 ml), and occasional agitation. Optical absorption was measured at 500 mμ in a Beckman Model-DU spectrophotometer. Similar measurements were made for the amino acids of the external and internal media and for amino acids released from the general protein after hydrolysis.

RESULTS AND DISCUSSION

In the accompanying report[1], the fractionation of preparations of hen-oviduct lipids by the process of paper chromatography in an acidic butanol solvent was described. It was shown that a fast-moving component possessed phenol–biuret-positive material and liberated the most ninhydrin-positive substances upon hydrolysis. Furthermore, free amino acids were absent from the material before hydrolysis, but were liberated by the process of hydrolysis. Fig. 1 shows that after a 5-min incubation with a mixture of radioactive amino acids the predominant radioactivity was located in the same area of the paper as was the phenol–biuret material and the source of ninhydrin-positive material. In this experiment, in order to avoid overloading the paper, the hydroxylamine-treated material from 10 hen oviducts was streaked on many paper sheets. Because of this, in the experiment depicted in Fig. 1 only a small additional amount of phenol–biuret-positive material over the background was present in the small aliquot used for chromatography. The results of many experiments, however, show the same pattern of radioactivity coincidental with the leading edge of a biuret-positive band (*e.g.*, see ref. 1). As a result of this procedure, much extraneous phenol–biuret-positive background material was contributed by the papers themselves. Fig. 2 shows the results of chromatography on Dowex-2 of the radioactive material eluted from the papers. In spite of the higher than normal background of biuret-positive material, it can easily be seen that the two sharp chromatographic entities described

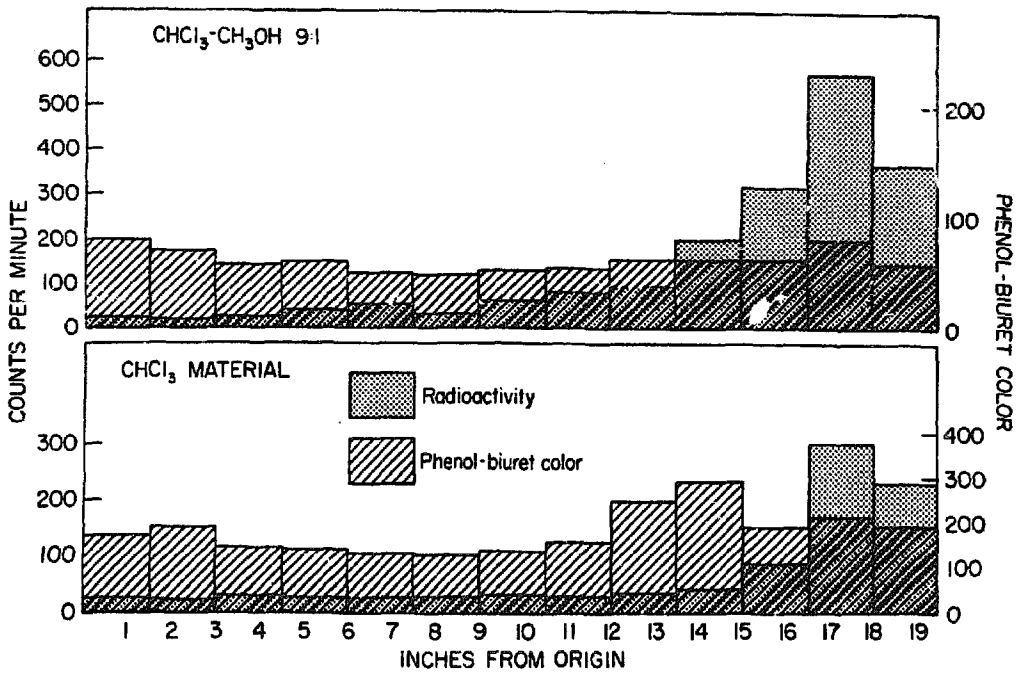


Fig. 1. Paper chromatograms of $CHCl_3$ and 9:1 materials*. The analyses were performed directly with 1.5-in strips numbered sequentially from the origin.

previously, A_1 and A_2, are encountered in their expected positions. Furthermore, it can be seen that the radioactivity was found in those peaks which upon hydrolysis liberated free amino acids and fatty acids as was shown previously[1]. Aliquots from the peaks were hydrolyzed and subjected to high-voltage electrophoresis to fractionate the free amino acids. Fig. 3 shows the distribution of radioactivity along the electrophoresis path in 1-in segments for hydrolysates from the A_1 and A_2 peaks from the chloroform and chloroform–methanol (9:1) eluates of silicic acid*. Radioactivity was found in the proper positions from the amino acids. Particularly striking, however, is the prominence of radioactivity in the No. 2 (*i.e.*, next to the origin) segments. This is the position for uncharged substances. From a series of other experiments it has been learned that this material gives a positive reaction with a benzidine sugar reagent[5] and that it passes through a Dowex-50 column in the acid form when eluted with water. Glucosamine and mannose are present in the glycoproteins of egg white and it was suspected that in the process of obtaining the free amino acids some artifact might be formed between these sugars and amino acids. To test this possibility, the electrophoretic pattern of an amino acid mixture was examined after the mixture was subjected to the same procedures used to obtain them experimentally. This was done both in the presence and absence of mannose and glucosamine. Fig. 4 shows that the materials in question are not formed by interaction between the sugars and the amino acids during isolation. From the hydrolysate of the radioactive tissue protein in this experiment it can be seen that similar radioactive "uncharged" moieties were present (Fig. 4, strip adjacent to the origin). This similarity between the composition of the protein hydrolysate and the hydrolysates of the A_1 and A_2 compounds is consistent with the possibility that these lipopeptides could be stages in the formation of the

* The chloroform eluate and the chloroform–methanol (9:1) eluate from silicic acid will be referred to as chloroform material and 9:1 material, respectively.

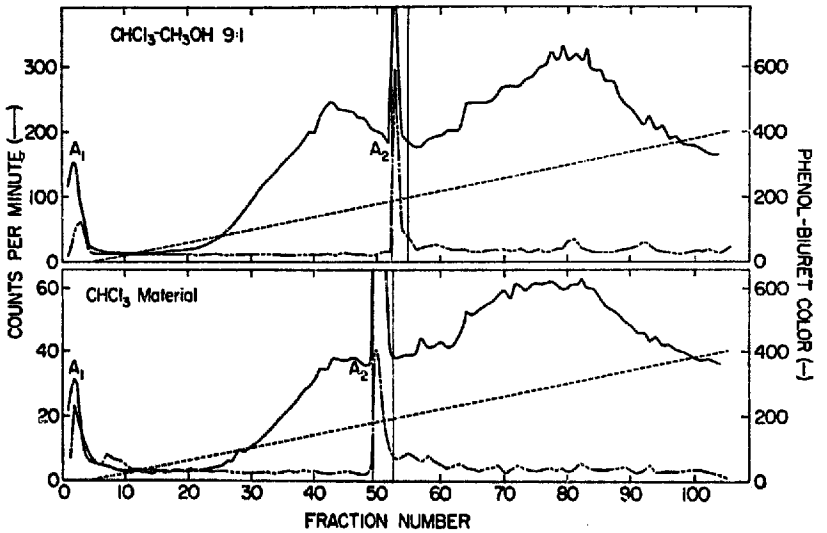


Fig. 2. Dowex-2 chromatography of radioactive material eluted from the paper chromatograms depicted in Fig. 1. The straight dashed diagonal line running from fraction No. 5 to the end represents the concentration of NH_4HCO_3 in the eluant from a value of 0 to a maximum of 0.5 M. The vertical lines on both sides of peak A_2 show where a dark brown tissue component was eluted.

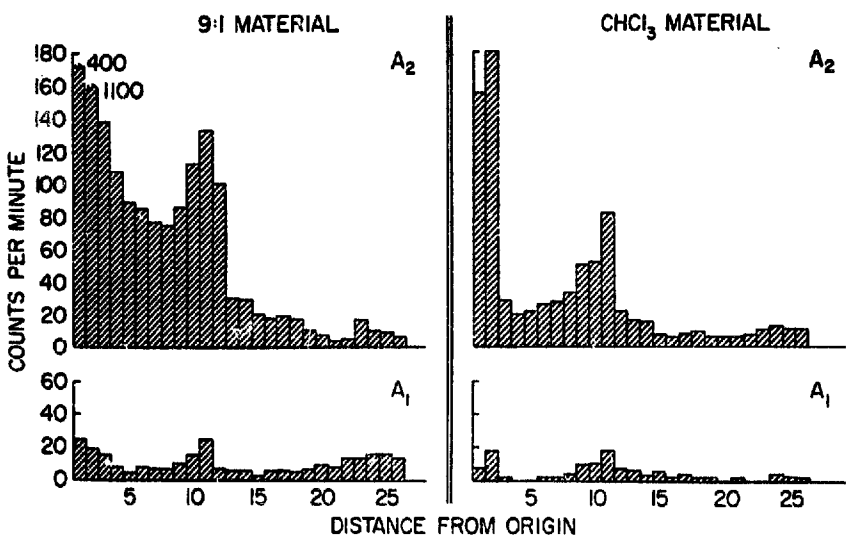


Fig. 3. High-voltage paper electrophoresis of hydrolyzed peaks A_1 and A_2 obtained from the Dowex-2 chromatography depicted in Fig. 2. The numbers on the abscissa refer to the 1.5-in sections which were individually analyzed. For relative positions of standard amino acids see Fig. 4.

protein. The amount of amino acid present in the strips whose radioactivity is depicted in Figs. 3 and 4 was directly determined. Table I lists the specific activities for leucine–isoleucine isolated from these various compounds. It should be noted that in the case of amino acids liberated from the lipopeptides, the numbers represent minimum levels because it was found that adjacent non-radioactive fractions from the Dowex-2 chromatography also had a small but significant liberation of unlabelled amino acid. Such a background contamination might serve to raise the ninhydrin values as much as 10–15 %. It was estimated that the lipopeptides A_1 and A_2 contained approx. 10–30 μmoles of amino acid per 100 g wet wt. of tissue[1]. The isotopic studies reported here suggest that after 5 min incubation with radioactive amino acids, these

materials contained 2–20 % of the amount of radioactivity that was found in the protein.

Table I shows that after a 5-min incubation with radioactive amino acids the specific activities of the amino acids in the "lipopeptides" were vastly greater than the specific activities of the amino acids found in the protein. Even after allowing for the fact that much of the protein present in the tissue is unlabelled stored protein, the great difference in specific activities is consistent with the idea that the lipopeptides represent precursor stages to the protein.

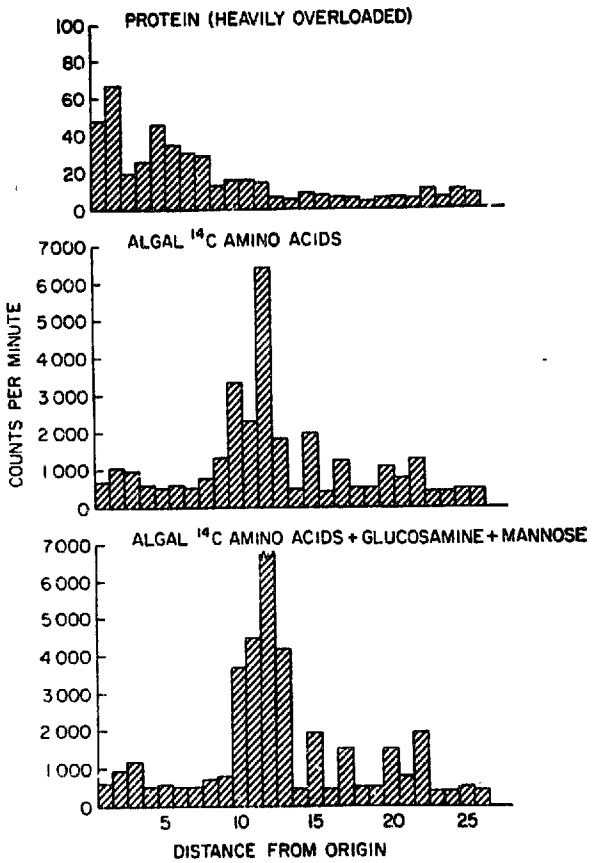


Fig. 4. High-voltage paper electrophoresis of amino acids obtained by hydrolysis from the protein of the same experiment depicted in Fig. 3 and from the ^{14}C-labelled algal hydrolysate used in this experiment with and without added carbohydrates. The numbers on the abscissa refer to the 1.5-in sections which were individually analyzed. For the algal hydrolysates, the amino acids were found in the numbered segments represented on the abscissa as follows: tyrosine, aspartic acid and phenylalanine at Nos. 8 and 9; glutamic acid at No. 10; proline and threonine at No. 11; leucine and isoleucine at No. 12; valine and serine at No. 13; alanine at No. 15; glycine at No. 17; arginine and histidine at No. 20; lysine at No. 22. For the amino acids in segments Nos. 8–13, there are various degrees of intermixing for adjacent amino acids.

An experiment was performed to confirm the specific activity differences just noted and to determine the kinetic state of the amino acids in the components represented by peaks A_1 and A_2 of the chloroform and chloroform–methanol (9:1) fractions from silicic acid. Four flasks were arranged as indicated in Table II. (All flasks contained 65 g hen-oviduct mince.)

After the pre-treatment (see Table II) ^{14}C-labelled algal hydrolysate was added to all flasks (0.345 mg containing $53 \cdot 10^6$ counts/min). After the initial 5 min incubation (see Table II) all flasks were plunged into ice and the mince was washed in iced unlabelled buffer. For flask No. 4 the buffer contained 10^{-3} M 2,4-dinitrophenol. The tissue in flasks Nos. 3 and 4 was re-suspended in fresh unlabelled buffer. The pre-treatment and subsequent incubations were carried out at 37°. In the discussion which follows, it should be remembered that a comparison of flasks Nos. 1 and 2 shows the effect of 2,4-dinitrophenol on the accumulation of radioactivity in the substances under study. A comparison of flasks Nos. 1 and 3 shows whether a radioactive

TABLE I

SPECIFIC ACTIVITIES OF LEUCINE–ISOLEUCINE FROM HYDROLYSATES OF VARIOUS COMPOUNDS

The amino acids were obtained by hydrolysis of the indicated compound and subsequent electrophoresis after the tissue was incubated for 5 min with a ^{14}C-labelled amino acid mixture. All figures are in counts/min/mg. The paper-strip numbers refer to the paper chromatograms depicted in Fig. 3.

Paper strip No.	*9:1 material*		*$CHCl_3$ material*		*Protein*
	A_2	A_1	A_2	A_1	
10	38 000	9 400	12 600	2500	9
11	37 000	10 600	13 300	2600	9

TABLE II

Flask No.	*Pretreatment, 10 min*	*Initial incubation, 5 min*	*Final incubation, 10 min*
1	No additions	No additions	
2	10^{-3} M 2,4-dinitrophenol	10^{-3} M 2,4-dinitrophenol	
3	No additions	No additions	100 mg casein hydrolysate
4	No additions	No additions	100 mg casein hydrolysate + 10^{-3} M 2,4-dinitrophenol

substance represents an end product or an intermediate capable of replacing its incorporated ^{14}C-labelled amino acids with ^{12}C amino acids. A comparison of flasks Nos. 1 and 4 shows the effect of dinitrophenol on the redistribution of radioactive materials during the final incubation in medium of reduced specific activities.

One other aspect of this experiment requires comment. The characteristics of the external and internal free amino acid pools differed in two striking respects. These were in the reduction of radioactivity that could be accomplished by replacing the medium (compare flask No. 3 to No. 1) and in the ratio of radioactivity in the acidic amino acids to that in the neutral amino acids, principally leucine and isoleucine (Fig. 6). These differences could be used in comparing the radioactivities of suspected metabolic intermediates so that equilibration of these compounds with the external and internal amino acid pools could be evaluated.

The effects of replacing the medium on the total and specific radioactivities of the free amino acid pools are shown in Fig. 5 (compare flask No. 3 to No. 1). The total radioactivity of the external pools was decreased 7.8-fold, whereas for the internal pool, the decrease was only 2.3-fold. For the average specific activity of the amino acids, tyrosine, aspartic acid, phenylalanine, glutamic acid, proline, threonine, leucine, isoleucine, serine, and valine*, the reductions were approx. 6.7-fold for the external and 2.5-fold for the internal medium. When average specific activities were measured in the total unfractionated media, the decrease was 10-fold for the external and only 1.1-fold for the internal medium. These data were obtained by assaying radioactivity and nin-

* Under the conditions of electrophoresis these amino acids occur sequentially and it is sometimes convenient to analyze them as a unit: tyrosine, aspartic acid, phenylalanine, glutamic acid, proline, threonine, leucine, isoleucine, serine and valine.

hydrin colour on the cold trichloroacetic acid-soluble fraction. Whereas the external medium had appreciable radioactivity in glutamic and aspartic acids, the permeability barriers to the dicarboxylic acids lessened the ratio of radioactivity between the acidic amino acids and leucine and isoleucine for the internal medium. This ratio was approx. 0.5 for the external medium and 0.25 for the internal medium (Fig. 6, compare external and internal free amino acid pools for flask No. 1). Fig. 5 shows the values for

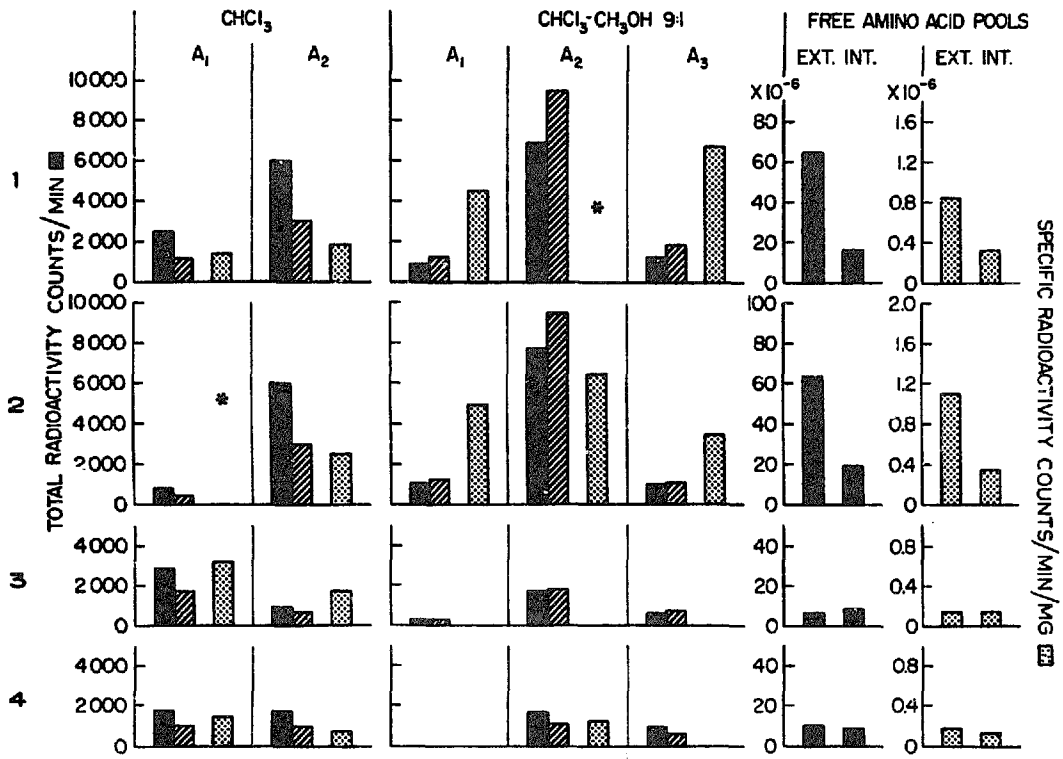


Fig. 5. Total and specific radioactivities for lipopeptide fractions, the total external pool of free amino acids and the total internal pool of free amino acids under four different experimental conditions. The numbers at extreme left (1–4) refer to the flask number for which complete details are described in the text. Total radioactivities are represented by solid bars or by bars with diagonal lines depending upon which one of two assumptions are made as explained in the text. The asterisks found above the specific activity bars for 2-$CHCl_3$ A_1 and 1-9:1 A_2 indicate that these two fractions were intermixed at this point and so the data is omitted. A_3 represents a diffuse band of radioactive material which was eluted from the Dowex-2 column after A_2 in this experiment. It was studied with the other compounds but no correlations are apparent at this time.

the total and average specific activities for the materials under study. Two values are shown for the total radioactivities from the chromatographic entities. That is a solid bar and a bar with diagonal lines. This is because two different assumptions have been made to obtain the total activities. Under the first assumption, the final pattern of radioactivities obtained in the Dowex-2 chromatography is taken as representative of the total radioactivity present in the stage before chromatography on paper (solid bars). Under the second assumption the Dowex-2 pattern is taken as representative of the total radioactivity eluted from the paper (bars with diagonal lines). Both assumptions appear equally likely at this point, since essentially all the radioactivity on the paper travelled in a single band. However, recoveries of radioactivity were incomplete for both the elution from the paper procedure (40–60 % recovery) and for the Dowex-2 stage (24–50 % recovery) and one or other of these assumptions is indicated.

The only differences introduced by assuming the second alternative over the first are that the increase of total radioactivity from flask 1-A_1 to flask 3-A_1 is somewhat larger and the decrease noted in the A_2 peak becomes relatively more significant for the 9:1 material than for the chloroform material (compare flask No. 1 to No. 3). The main

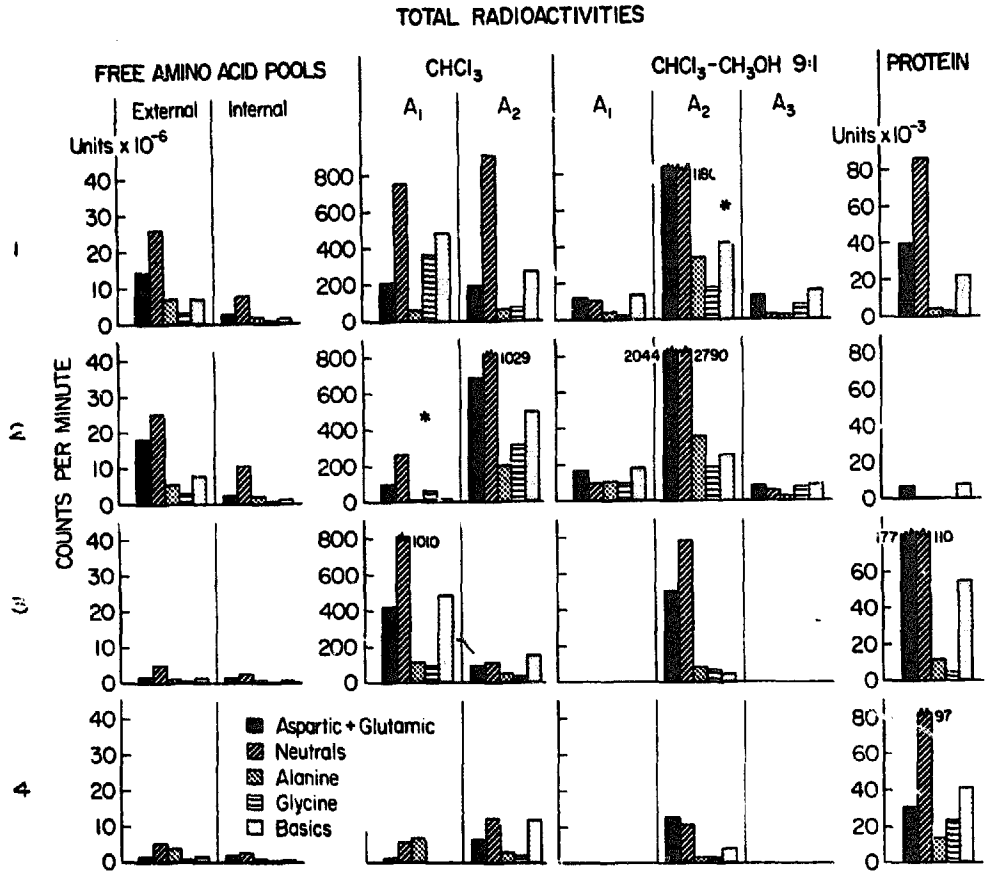
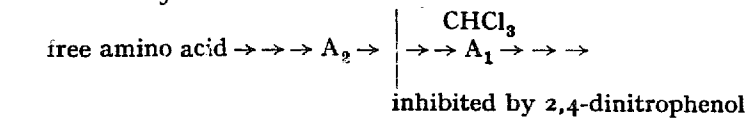


Fig. 6. Total radioactivities for individual amino acids and groups of amino acids obtained from hydrolysates of the lipopeptide fractions, the external and internal amino acid pools, and the protein. The numbers at the extreme left (1–4) refer to the flask number for which complete details are given in the text. The "neutrals" refer mainly to proline, threonine, leucine, isoleucine, serine and valine. The "basics" include arginine, histidine and lysine. For an explanation of the asteriks and A_3 see Fig. 5.

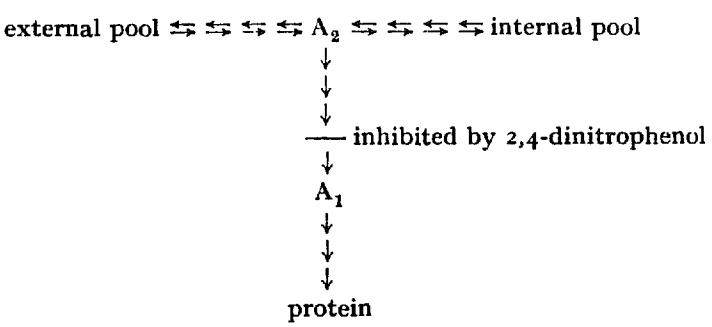
results are unaffected, however. That is it can be seen that the A_2 material of both fractions markedly drops in radioactivity as a result of lowering the radioactivity of the amino acid pools. The chloroform A_1 material, on the other hand, loses no activity in the process, but instead, gains in activity (compare flask No. 1 to No. 3). The uptake of radioactivity into the A_2 materials (both from the $CHCl_3$ and 9:1 fractions) is not inhibited by the presence of 2,4-dinitrophenol, whereas the uptake of radioactivity into the chloroform A_1 peak is seriously inhibited (compare flask No. 2 to No. 1). The behavior of the 9:1 A_1 peak is sufficiently different from that of the $CHCl_3$ A_1 peak that it is considered to be a different entity. During the phase of incubation in the medium of lower specific activity, radioactivity appears as though it passes from A_2 to A_1 of the chloroform material in the absence of 2,4-dinitrophenol (compare flask No. 1 to No. 3), but in the presence of this poison, although radioactivity leaves A_2,

none appears in A_1 (compare flask No. 1 to No. 4). From these considerations the following sequence seems likely:



It may be recalled that 2,4-dinitrophenol inhibited the flow of radioactivity from a more polar supernatant component into a less polar cell debris component[3]. From Fig. 2 it can be seen that A_2 is present in greater relative amount in the 9:1 material than in the chloroform material and so the 2,4-dinitrophenol block would appear to be in the flow from A_2 to $CHCl_3$ A_1.

The relative decrease of radioactivity for either the chloroform or 9:1 A_2 peak under either assumption is intermediate in value for the decrease of radioactivity of the external and internal pools (Fig. 5). Furthermore, from the electrophoresis distribution of the hydrolysates of the 9:1 A_2 materials it was found that a distinct radioactive peak for glutamic acid was present, and the ratio of radioactivity in the acidic amino acids to that in leucine–isoleucine was approx. 0.7 (flasks Nos. 2 and 3). This value is quite close to the situation in the external medium as opposed to the internal medium (flasks Nos. 1 and 3). It, therefore, seems as though the A_2 material especially of the 9:1 fraction had access to the external as well as the internal medium. The chloroform A_1 material on the other hand, showed no distinct peak for glutamic acid radioactivity upon electrophoresis and the ratio of radioactivity between the acidic amino acids and leucine–isoleucine was approx. 0.3 for flask No. 1 and slightly higher for flask No. 3 (Fig. 6). Finally, it can be seen (Fig. 6) that relative distributions for radioactivity in the different amino acids from the hydrolysates of the chloroform flask 3-A_1 and the protein were quite similar. On the basis of these considerations, the following scheme is being considered:



In support of this idea is the increase in the specific activities noted for the free amino acids of the internal pool and the occurrence of high specific activities for the amino acids in the hydrolysates of the A_2 materials in the presence of 2,4-dinitrophenol (flask No. 2, Fig. 6). The scheme in its present form is undoubtedly an oversimplification. If A_2 is a peptide, a less complex component must exist before A_2 to serve as an amino acid carrier. The similarity of this representation to one recently proposed[2] is apparent. Table III lists the average specific activities for the group of amino acids listed earlier* and for leucine–isoleucine alone isolated from the hydrolysates of all

* Under the conditions of electrophoresis these amino acids occur sequentially and it is sometimes convenient to analyze them as a unit: tyrosine, aspartic acid, phenylalanine, glutamic acid, proline, threonine, leucine, isoleucine, serine and valine.

TABLE III

SPECIFIC ACTIVITIES OF AMINO ACIDS FROM VARIOUS COMPOUNDS

The numbers at the extreme left (1–4) refer to the experimental conditions as explained in the text for flasks with the corresponding numbers. All figures represent counts/min/mg. W, insufficient material for accurate specific activities; O, sample lost.

		$CHCl_3$ material		*9:1 material*			*Protein*
		A_1	A_2	A_1	A_2	A_3	
1	Leu–Ileu	4090	3150	4500	6670*	2580	93
	Average	1330	1807	4570	3370*	6750	38
2	Leu–Ileu	4100*	3500	5620	32 900	3500	0
	Average	4680*	2470	4990	11 410	3440	1.2
3	Leu–Ileu	7800	2330	W	O	W	200
	Average	3210	1700	W	O	W	71
4	Leu–Ileu	4080	1000	W	1440	W	100
	Average	1440	810	W	1250	W	53

* 1–9:1 A_2 cros-scontaminated with 2-$CHCl_3$ A_1.

the compounds under study. These values for specific activities confirm the fact that the amino acids of the lipopeptides are very much more radioactive than those obtained from the proteins after a short-term incubation. Also shown is the fact that replacing the ^{14}C-labelled amino acids with ^{12}C amino acids results in an increase of radioactivity for the amino acids of $CHCl_3$-A_1 and a decrease for A_2 (flasks Nos. 1 and 3). The studies reported in this paper lend further support to the idea that amino acids may enter a lipid phase for the early stages of protein synthesis.

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