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ISOLATION OF "LIPOPEPTIDES" FROM HEN OVIDUCT

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SUMMARY

The isolation of two apparent lipopeptides from hen oviduct is described. It was found that fatty acids and amino acids were liberated from these compounds by hydrolysis. Arachidonic acid was particularly prominent in the hydrolysate of one, and palmitic acid in the hydrolysate of the other. The isolation serves as a basis for further metabolic experiments which are described in an accompanying paper.

INTRODUCTION

A recent report from this laboratory described characteristics of partially purified lipid-amino acid preparations from hen oviduct in terms of a possible role of such compounds in the metabolism of the amino acids[1]. Evidence was presented for specificity in the selection of amino acids and for the covalence of bonding of both the amino and carboxyl groups of the amino acids in such compounds. Observations which suggested the ready formation of hydroxamic acids of the amino acid components and the rapid incorporation of amino acid into these compounds were also presented. The present report describes the further purification of this material, presents evidence that the amino acids occur in the form of a peptide or peptides, and that particular fatty acids appear to be a part of the molecule.

MATERIALS AND METHODS

Source of tissue

An ether extract of 60–70 oviducts was obtained from Dr. G. ASHWELL of the National Institute for Arthritis and Metabolic Diseases*.

Purification procedure

Fig. 1 outlines the general scheme of isolation for the lipopeptides described in this paper. Details of the procedure up to the paper-chromatography stage, have been published[1-3]. The only change in the previously published procedure is that chloroform–ethanol (9:1) has been found to be a more effective solvent for the extraction of the hydroxamates than ethyl alcohol alone.

* The author is indebted to Dr. G. ASHWELL for this gift. The details for the source of the oviducts and preparation of the ether extract have been described[12].

GENERAL ISOLATION SCHEME FOR "LIPO-PEPTIDES"

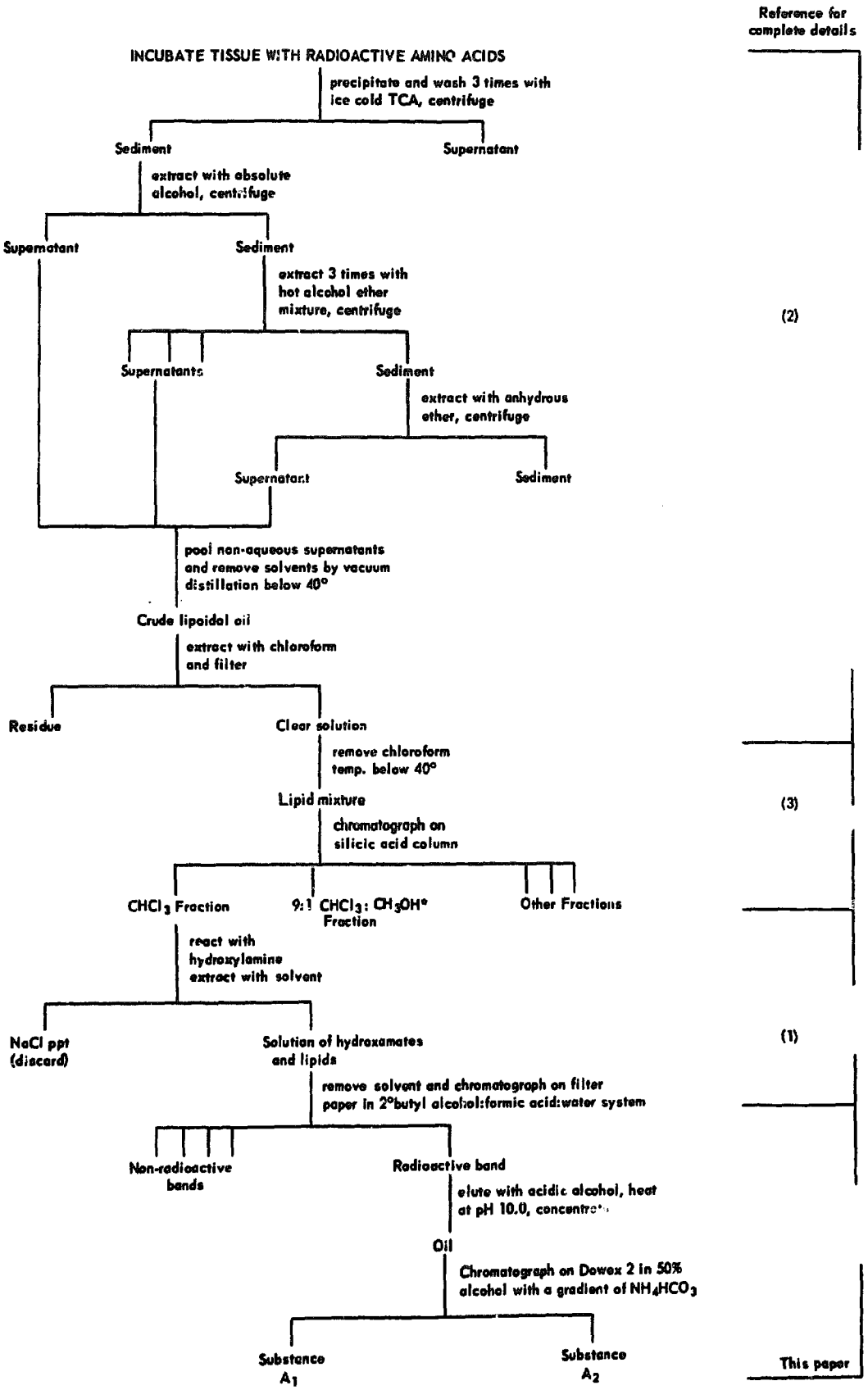

Fig. 1.

Chromatography in sec.-butyl alcohol–formic acid–water (75:15:10) system

The hydroxamates were streaked on Whatman 3MM paper and developed by descending chromatography into a series of bands. Narrow strips were cut from each end in order to locate various types of compounds.

Location of hydroxamate-positive material

The paper strips were dipped into a solution which was prepared as follows: 3.3 ml of 30% $FeCl_3 \cdot 6H_2O$ and 0.1 ml concentrated HCl were made to 100 ml with acetone. Hydroxamates were revealed as colored bands of orange, red or purple hue.

Location of phenol–biuret positive material

0.5-in strips were cut into 1-in sections and each 0.5 × 1-in section was placed in a test tube. The reagent was made by adding 4 ml of a 0.5% $CuSO_4$ in 1% sodium–potassium tartrate solution plus 40 ml of 0.5 N NaOH to 200 ml of a 2% Na_2CO_3 solution. 6 ml of this reagent was added to each test tube and after 10 min 0.45 ml of Folin–Ciocalteau phenol reagent (diluted to 1 N) was added. The solutions from the tubes after standing 30 min were measured for light absorption at 660 mμ in a Coleman, Jr. spectrophotometer or with a No. 66 filter in a Klett Summerson spectrophotometer.

Determination of ninhydrin-positive material before and after hydrolysis

The rest of the chromatogram was cut into 1-in strips, numbered sequentially from the origin and these were eluted with water. Aliquots of the eluates were taken for hydrolysis in 6 N HCl for 16 h at 120°. The hydrolysates were evaporated to dryness. Residual acid was removed by adding water and evaporating to dryness three more times. Ninhydrin analyses were performed on corresponding hydrolyzed and unhydrolyzed aliquots. After water elution of the strips, it was observed that strips Nos. 16–19 still had a brownish material present. These strips were eluted clean with absolute ethanol. Ninhydrin analyses before and after hydrolysis were also performed on these eluates.

Preparation of ion-exchange material for chromatography

Analytical-grade anion-exchange resin Bio-Rad AG 2-X8 (Dowex-2) 200–400 mesh, chloride form, was washed by occasionally swirling in 0.5 M NH_4HCO_3, in an Erlenmeyer flask during a 15-min interval. The supernatant solution which contained fine particles was removed by decantation. This procedure was repeated 3 times and then successive washes were employed as follows: two times with 1 M NaOH–absolute ethanol (1:1) and finally 50% ethanol to remove excess NaOH. The resin was allowed to stand in 50% alcohol for a period of 1–4 days during which time it acquired a translucent appearance. The water and alcohol eluates obtained from the paper chromatograms as described above were individually adjusted to pH 10 with concentrated NH_4OH and evaporated to dryness with a boiling-water bath. Water was then added to each of these and the evaporation repeated to remove excess ammonia. The purpose of this operation was to hydrolyze any easily hydrolyzable bonds (*e.g.*, hydroxamic acids) so that heterogeneity due to spontaneous hydrolysis should not complicate the chromatography on Dowex-2. Approx. 2.3 ml of column volume was

employed for each 1000 absorbancy units of phenol-biuret color. Column length was approx. 25 times the diameter.

Chromatography on Dowex-2

With 50 % alcohol as the elutant, and a constant head of about 120-in of water, five fractions were collected with a total volume of approx. 1 column volume. The column was then eluted with a linear gradient of NH_4HCO_3 in 50 % alcohol set up as follows: a mixing chamber containing approx. 10 column volumes of 50 % alcohol was connected to the column and also to a reservoir comprised of an identical bottle containing an equal volume of 0.5 M NH_4HCO_3 in 50 % alcohol[4]. Both the mixing chamber and reservoir were maintained under a constant head of 120-in of water pressure and the height of liquids in both bottles decreased simultaneousiy.

Analysis of Dowex-2 column fractions

Aliquots were taken from each fraction for phenol-biuret analysis[5], and for hydrolysis in 6 N HCl 16 h at 120°. Prior to hydrolysis, most of the NH_4HCO_3 was decomposed by evaporation to dryness in a boiling-water bath under an atmosphere of nitrogen. The process was repeated two more times with the addition of small quantities of 50 % alcohol to the residue. The acid hydrolysates were evaporated to dryness as previously described and then taken up in 0.5 M HCl for extraction of fatty acids in isooctane. The fatty acids were titrated and then analyzed by gas chromatography*. The aqueous fraction was evaporated to dryness and the small remaining quantities of ammonium chloride removed by heating in a vacuum oven at approx. 150° for 1–2 h. The residue was then subjected to high-voltage electrophoresis essentially as described previously[3]. The separation was carried out at 5000 V in specially constructed tanks[6],**.

Synthesis of palmityl amino acids

Approx. 5 μg of radioactive amino acid (specific activity $2 \cdot 10^5$ counts/min/μg) in a volume of 10 μl was introduced into and dried in a 25-ml Erlenmeyer flask. 1 mmole of dry unlabelled amino acid was added to the flask and then 5 ml of ACS, analyzed reagent pyridine and 0.3 ml of palmitoyl chloride (0.98 mequiv). The flask was stoppered and placed on a shaker. The palmitoyl chloride is insoluble in pyridine, but dissolves slowly as the reaction proceeds. After 1 h it was completely dissolved, but shaking was generally continued for several hours longer. The reaction mixture was filtered and approx. 2 ml of pyridine was used to rinse the reaction flask and filter paper. The pyridine was evaporated off under a stream of nitrogen while warming the solution with infrared lamps. The residue was recrystallized several times from alcohol–water mixtures. By this procedure, palmityl derivatives were made from valine, glutamic acid, glycine and leucine–isoleucine mixture. Elemental analysis for palmityl valine: m.p. 47–48°; Found: C, 71.75 %; H, 11.47 %; N, 3.68 %; Calculated for $C_{21}H_{41}O_3N$: C, 71.0 %; H, 11.52 %; N, 3.94 %. Elemental analysis for palmityl leucine–isoleucine. Found: C, 71.35 %; H, 11.39 %; N, 3.60 %; Calculated for

* The author is indebted to Dr. A. KARMEN of the National Heart Institute for the gas-chromatographic analysis reported in this paper.

** The tanks were constructed by Gilson Medical Electronics, Middleton, Wisc., in collaboration with Dr. W. J. DREYER.

$C_{22}H_{43}O_3N$: C, 71.5%; H, 11.63%; N, 3.79%. Upon partioning a mixture of [^{14}C]-palmityl amino acids between chloroform and water, it was found that 96% of the radioactivity was extracted into the chloroform.

RESULTS

Fig. 2 shows the results of analysis of a paper chromatogram of hydroxylamine-treated lipid-amino acid material which was obtained in the chloroform fraction* from silicic acid column chromatography[1]. The paper chromatogram was analyzed in the form of 1-in sections, cut and numbered sequentially from the origin. It can be seen that the location of the major phenol-biuret-positive material occurred in the broad band which had a peak 17-in from the origin. The most prominent nin-

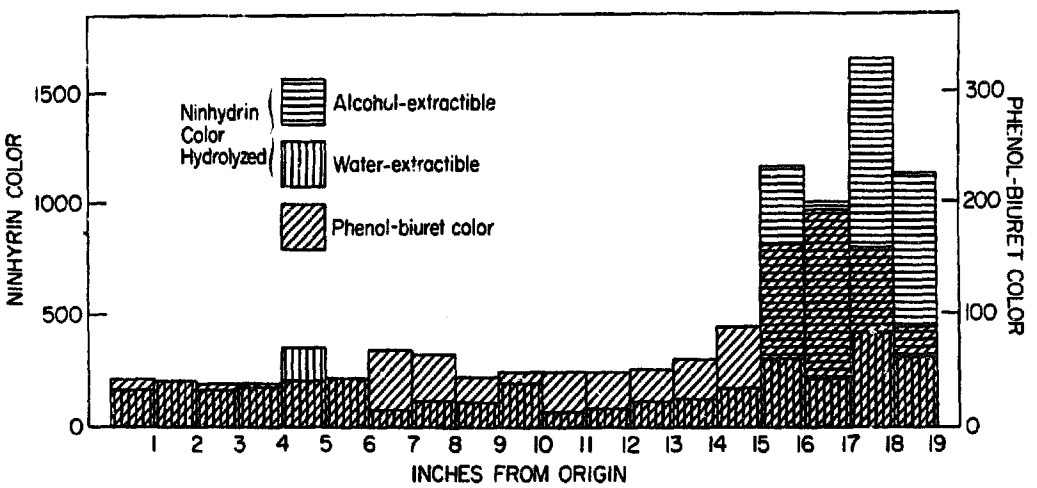

Fig. 2. Analysis of paper chromatogram of hydroxylamine-treated lipids of the chloroform material*. 1-in sections numbered sequentially from the origin were individually analyzed as described in the text. The units for the two ordinates refer to readings with a Klett Summerson spectrophotometer. For ninhydrin color No. 56 filter was used and for phenol–biuret a No. 66 filter.

hydrin-positive areas after hydrolysis were found in segments Nos. 16–19 with peaks at 16-in and 18-in. It can also be seen that much more ninhydrin-positive material after hydrolysis was present in the alcohol extract than in the water extract. One interesting observation that tended to obscure the picture was that the alcohol eluate contained a substance which gave a high ninhydrin reading even before hydrolysis. The color appeared a bit more red, however, than that from amino acid, and as will be described below, no free amino acids were present in the unhydrolyzed material. Hydrolysis always increased the ninhydrin positive value and resulted in the liberation of amino acids. Not shown in the figure is the fact that the ferric chloride dip revealed an orange band at strip No. 5, a purple-red band at No. 8 and a diffused purple-red band at Nos. 17–19. More concentrated extracts gave many more colored bands of orange, red or purple hue. The eluates from the 1-in strips cut from this paper chromatogram were subjected to high-voltage paper electrophoresis before and after hydrolysis. Reference amino acid mixtures were simultaneously analyzed. It was

* This material obtained from silicic acid in the chloroform fraction will be referred to as chloroform material, whereas the fraction obtained with chloroform–methanol (9:1) will be referred to as 9:1 material.

found that after hydrolysis complete amino acid mixtures were present in the eluates of strips Nos. 16–19. The hydrolysates of the other strips had either none or barely detectable amounts of amino acids. Furthermore, before hydrolysis no amino acids were present in the eluates of strips Nos. 16–19.

Further fractionation of the material eluted from the paper chromatograms was achieved by use of a basic resin which contained an organic hydroxyl group (Dowex-2) and which was employed in an aq. alcoholic medium. Fig. 3a (middle) shows the result of chromatography with a mixture of an aliquot of the aqueous eluate from the paper chromatogram which contained 1750 absorbancy units of phenol–biuret material together with an aliquot of the alcohol eluate from the paper chromatogram which contained 13200 absorbancy units of phenol–biuret material. The pattern obtained in this case was identical to a previous fractionation performed with the water eluate alone. It therefore seems that the same material which was efficiently eluted from the paper chromatograms with ethanol was eluted less efficiently with water. Fig. 3b (bottom) shows the results obtained with a similar mixture of the two eluates in different proportions. From Fig. 3, it can be seen that two sharp phenol–biuret-positive peaks emerged. One of these appeared before the gradient was applied and the other just before the midpoint of the linear gradient. These two peaks will be referred to as A_1 and A_2, respectively. The fatty acid (μmoles) released by hydroly-

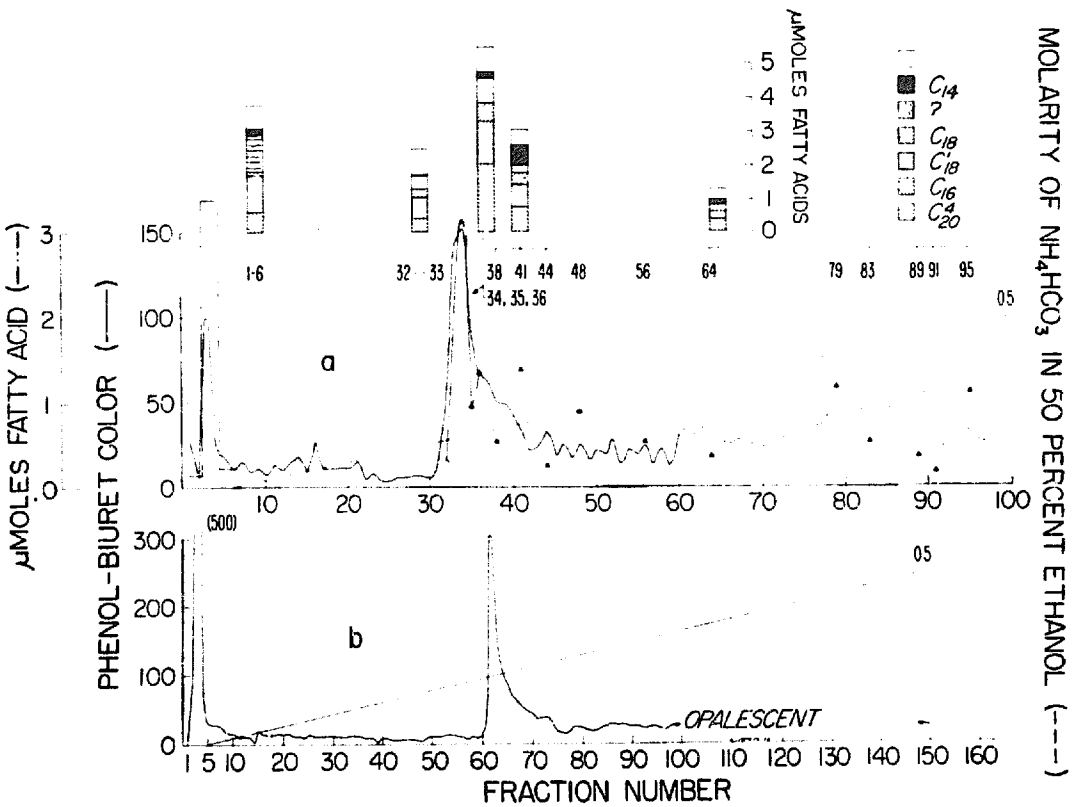

Fig. 3. Dowex-2 chromatography of peak material eluted from paper chromatogram depicted in Fig. 2. Shown in Figs. 3a and 3b are two fractionations with mixtures which had different relative proportions of water and alcohol eluates from the paper chromatograms. The bars at the top represent the composition of fatty acid mixtures obtained by hydrolysis of the indicated pooled fractions. The white portion of these bars represents other non-specified fatty acids present in smaller amounts. The solid triangles and the numbers directly above represent individual tubes selected for fatty acid analyses.

sis is shown in Fig. 3a as a dash-dot line and triangular points. The peaks for fatty acid-release coincided with the peaks for phenol–biuret-positive material. The results of gas chromatographic analysis are shown at the top of the figure for pooled samples from these peaks and for other fractions. Peak A_1 was unique in having an unusually high content of arachidonic acid and peak A_2 showed a considerable enrichment both for percentage and absolute amount of palmitic acid.

Aliquots from individual tubes, before and after hydrolysis, were subjected to high-voltage paper-electrophoretic analysis. It was found that the fractions in the A_2-peak tubes liberated complete mixtures of amino acids as a result of hydrolysis. Before hydrolysis no free amino acids were present. There was liberation of amino acids in much smaller amounts from some of the other fractions. From specific-activity measurements of amino acids liberated from the substances A_1 and A_2 after hydrolysis of material obtained from incubations with ^{14}C-labelled algal amino acids; and from total radioactivities of the same material, some quantitation can be inferred[7]. Each of these peptides from the $CHCl_3$ and 9:1 material contains approx. 10–30 μmoles of amino acid per 100 g of fresh tissue. As more purified material is obtained stoichiometric relations between the various components of the molecule will be studied.

The presence of a variety of different amino acids after hydrolysis of a material that has been purified by the combination of several different chromatographic procedures strongly suggests that they are a part of the same molecule. FUKUI AND AXELROD described the presence of palmityl amino acids in rat liver[8]. The enrichment of palmitic acid in material A_2 (Fig. 3a) suggested the possibility that a mixture of individual palmityl amino acids might be fractionated principally on the basis of the large aliphatic part of the molecule. To test the idea that such a mixture might not be separated by this chromatographic procedure on Dowex-2, a mixture of palmityl derivatives of radioactive glycine, valine, glutamic acid, leucine and isoleucine was prepared and subjected to chromatography on Dowex-2. It was found that the material started to emerge sharply about the middle of the gradient and continued to emerge at the same rate throughout the whole column development. High-voltage electrophoresis of hydrolysates of all of the fractions showed that no resolution took place and that all five amino acids were present in all fractions. These results demonstrate that the natural product is not a mixture of individual palmityl amino acids and that the presence of the large aliphatic chain in a mixture of smaller molecules containing amino acids is not a strong enough factor in itself to dominate the behaviour of these compounds upon purification.

DISCUSSION

The material described in this paper has been referred to as "lipopeptide". The basis of this designation is that: (a) it behaves as a single entity during several different types of chromatographic fractionations; (b) no free amino acids are present before hydrolysis, but many are liberated by hydrolysis; (c) the amino acids present appear to have both the amino and carboxyl groups involved in covalent bonds, and (d) the demonstration that a mixture of palmityl amino acids are extensively spread out by the chromatographic procedure that causes two single sharp peaks of the natural substances to emerge. The quotation marks are employed because the final

characterization must await the derivation of a complete structural formula. The predominance of palmitic acid from peak A_2 is especially interesting in light of the demonstration of FUKUI AND AXELROD[8] of an enzyme in rat liver which causes the formation of individual palmityl amino acids. The fact that palmitic acid remained in this compound after treatment with hydroxylamine suggests that it is bound to the amino end of an amino acid in the form of an amide linkage as opposed to an ester linkage with some other hydroxyl group in the molecule. If indeed, the material is a peptide-containing palmitic acid, it is interesting to consider that palmitic acid may participate in the actual process of linking the amino acids together to form a peptide in the lipid stage. That is, palmityl amino acids may be condensed in the lipid phase to form palmityl peptides. There is no direct evidence at present on the nature of the material which is linked to the carboxyl end of the amino acid. However, in view of persistant reports from the laboratory of Dr. E. GALE on the existence of an amino acid incorporation factor which is related to glycerol[9], and because of the apparent ease with which the compounds form hydroxamic acids, glycerol or a derivative of it, is suspected as the material involved in this position. Phosphatidyl glycerol amino acid esters have been recently identified in extracts of bacterial cell membranes[10]. Recent experiments have shown that phosphate is contained in the lipopeptide material described in this paper and that the phosphate-containing moiety is split off during the reaction with hydroxylamine[11]. In the following paper[7], evidence is presented which shows that the amino acids in the compounds discussed in this paper are in a very rapid state of metabolic flux.

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