acetylene in 100 ml. of carbon tetrachloride was heated at 45° for 114 hr. The solvent was removed under reduced pressure and the orange-brown residue was hydrolyzed in 50 ml. of a 3% hydrochloric acid solution of 50% aqueous dioxane at 25° for 3 hr. and at reflux for 14 hr. Dilution with water afforded a dark solid which upon trituration with 80% ethanol yielded 0.092 g. (32%) of crude anisil, m.p. 120–123°. Chromatographic purification on silica gel and recrystallization from ethanol afforded material of m.p. 129.5–131°, mixed m.p. 129.5–131.5°. Extraction of the aqueous hydrolysis layer with ethyl acetate, removal of solvent, and trituration with chloroform afforded 0.29 g. (76%) of phthalic acid, identified by sublimation to phthalic anhydride.

Reaction of Phthaloyl Peroxide with Tetraphenylethylene.—A solution of 0.42 g. (2.56 mmoles) of phthaloyl peroxide and 0.85 g. (2.56 mmoles) of tetraphenylethylene in 125 ml. of carbon tetrachloride was heated at reflux for 15.5 hr. Solvent was removed from the yellow-orange solution under reduced pressure. The residue showed strong absorption in the infrared at 1780 cm.$^{-1}$. The residue was hydrolyzed in 40 ml. of 75% aqueous ethanol containing 4.5 ml. of concd. sulfuric acid for 12 hr. at reflux. A light tan solid, 0.80 g., precipitated on cooling and was chromatographed on 22 g. of basic alumina. Elution with 19:1 petroleum ether–benzene yielded 0.325 g. of tetraphenylethylene of m.p. 221–222.5° after one recrystallization from benzene; mixture m.p. showed no depression. Elution with 1:1 petroleum ether–benzene gave 0.219 g. of benzpinacolone of m.p. 180–181° after one recrystallization from benzene–ligroin; mixture m.p. showed no depression. Attempts to obtain evidence for benzophenone were unsuccessful.

Subjection of the crude olefin–peroxide reaction mixture to basic hydrolysis and work-up by the method described above afforded phthalic acid in 60% yield, characterized by conversion to the anhydride.

Reactivity of Phthaloyl Peroxide with Various Substrates.—Six-ml. portions of a solution of phthaloyl peroxide (0.00945 *M*) and substrate (0.015 *M*) in carbon tetrachloride were sealed in Pyrex tubes and heated for the requisite time intervals at 80°. Consumption of peroxide was followed by iodometric analysis of 5-ml. aliquots. The data are summarized in Table III.

Kinetics.—The rates of reaction of phthaloyl peroxide with the unsaturated compounds were followed by iodometric analysis for peroxide by the methods described previously.[3] The data are summarized in Fig. 1 and in Tables I, II, and IV.

TABLE IV

RATE OF REACTION OF DI-*p*-METHOXYPHENYLACETYLENE WITH PHTHALOYL PEROXIDE IN CARBON TETRACHLORIDE AT 80°[a]

Time, sec. × 10^3	Thiosulfate soln.,[b,c] ml.
0	12.09
0.36	11.40
2.04	7.50
3.24	5.40
3.84	4.71
5.22	3.71
6.78	2.94

[a] k_2 (graphical) = 11.8 × 10^{-3} M^{-1} sec.$^{-1}$. Initial concentrations, 0.0120 *M*. [b] 0.00996 *N*. [c] Five-ml. aliquots of reaction solution.

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[CONTRIBUTION FROM THE CLAYTON FOUNDATION BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

Studies on the Nature of Protein-bound Lipoic Acid

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Highly purified pyruvate and α-ketoglutarate dehydrogenation complexes (from *Escherichia coli*) containing bound radioactive lipoic acid were oxidized with performic acid and then partially hydrolyzed with 12 *N* hydrochloric acid (3 hours at 105°). From the hydrolysates was isolated in good yield a ninhydrin-positive, radioactive conjugate which was identified as ε-N-(6,8-disulfoöctanoyl)-L-lysine by degradation and synthesis. The lipoyl moiety in the two complexes therefore is bound in amide linkage to the ε-amino group of a lysine residue. Partial hydrolysis of the oxidized pyruvate dehydrogenation complex under milder conditions (7 days at 37°) yielded three radioactive 6,8-disulfoöctanoylpeptides. Analysis of these peptides indicated that the amino acid sequence about the lysine residue is either Ala.Lys.Asp. or Asp.Lys.Ala.

Early studies on the distribution of lipoic acid indicated that it occurs in tissues largely in association with proteins. Thus, it was not extractable by hot water or by lipid solvents but was released by hydrolysis with acid, alkali or crude proteolytic enzymes.[2–4] Subsequent studies revealed that "bound" lipoic acid is an integral part of CoA- and DPN-linked pyruvate and α-ketoglutarate dehydrogenation systems.[5–9] An investigation of components and conditions required for incorporation[7] of lipoic acid into bacterial apopyruvate dehydrogenation systems and for its release[8] from the holoenzymes indicated that lipoic acid is bound to protein in covalent linkage through its carboxyl group. This paper presents evidence that lipoic acid is bound to the ε-amino group of a non-terminal lysine residue. A preliminary report of this work has appeared.[10]

When *Escherichia coli* is grown aerobically in the presence of lipoic acid-S_2^{35}, the latter substance is incorporated into the pyruvate and α-ketoglutarate dehydrogenation systems. These systems have been isolated as structural units (en-

(1) Rosalie B. Hite Postdoctoral Fellow, 1958–1959, while on leave from Takeda Pharmaceutical Industries, Osaka, Japan.

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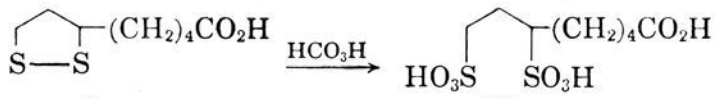
zyme complexes) of high molecular weight.[11,12] Preliminary attempts to isolate radioactive fragments from acid hydrolysates of the enzyme complexes did not show promise, due presumably to susceptibility of the lipoyl moiety to oxidation, polymerization and disulfide interchange.[15,16] To avoid these complications, the radioactive complexes were oxidized with performic acid[17] prior to acid hydrolysis. It was first established that treatment of lipoic acid (I) with performic acid converted the former substance to 6,8-disulfooctanoic acid (II). The oxidized protein was



I II

subjected to various conditions of acid hydrolysis and the hydrolysates were examined by means of paper electrophoresis and paper chromatography. The hydrolysate obtained by heating the oxidized protein with 12 *N* hydrochloric acid for 3 hr. at 105° contained two major radioactive substances, as revealed by radioautography. These two substances were isolated by passing the hydrolysate through a column of Dowex 50W-X8 in the hydrogen cycle, followed by paper electrophoresis and then paper chromatography. Essentially all of the radioactive material emerged unretarded from the Dowex 50 column, indicating that the two radioactive substances were strongly acidic.[18,19] A major portion of the ninhydrin-positive material in the hydrolysate was retained on the column. All of the radioactive material in the effluent migrated toward the anode during paper electrophoresis in 1 *N* acetic acid, *p*H 2.3.[18] Partial separation of the two radioactive substances was achieved by this procedure. Further purification was achieved by descending paper chromatography with butanol–acetic acid–water (2:1:1) as the solvent system (Fig. 1). Homogeneity of the two radioactive substances was established by subjecting samples to paper chromatography and paper electrophoresis. The faster-moving radioactive substance was ninhydrin-negative and migrated at the same rate as authentic DL-6,8-disulfooctanoic acid-S_2^{35}. The slower-moving radioactive substance gave a color immediately with ninhydrin at room temperature. The recovery of radioactivity in the conjugate amounted to 47 and 60% in two isolations. In the latter isolation 20% of the original radioactivity was recovered as 6,8-disulfooctanoic acid.

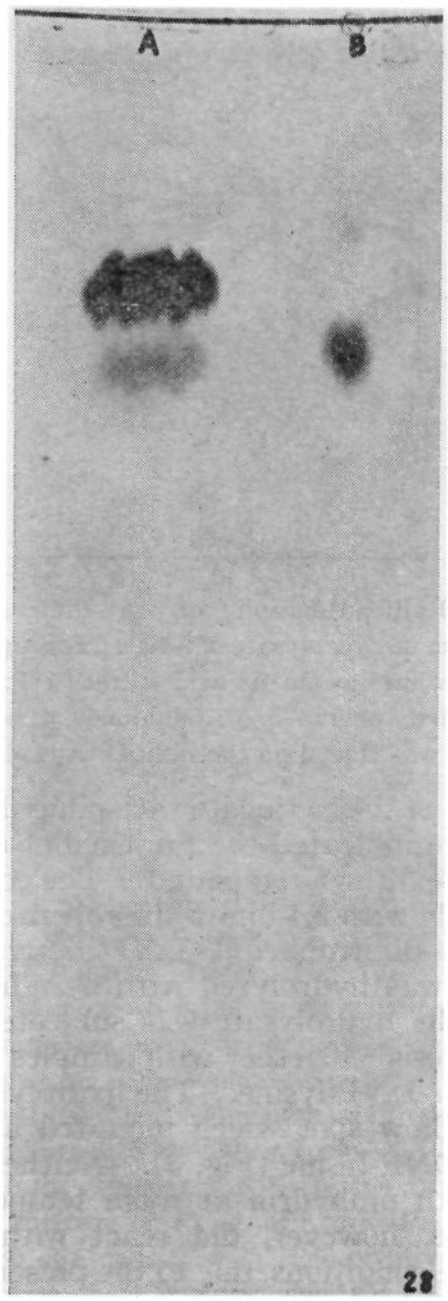


Fig. 1.—Radioautograph of one-dimensional paper chromatogram of purified fraction from partial hydrolysate of oxidized pellet: A, fraction from electrophoresis run; B, 1.5 μg. of DL-6,8-disulfooctanoic acid-S_2^{35}. Chromatogram was developed with butanol–acetic acid–water (2:1:1) for 24.5 hr.

A sample of the radioactive conjugate was hydrolyzed with 6 N hydrochloric acid and the hydrolysate was subjected to two-dimensional paper chromatography. A ninhydrin-negative, radioactive spot and a ninhydrin-positive, non-radioactive spot were detected on the chromatogram (Fig. 2). These spots appeared to be 6,8-disulfooctanoic acid-S_2^{35} and lysine, respectively, as indicated by comparative paper chromatography with authentic samples. Microbiological assay of the hydrolysate showed the presence of L-lysine in an amount corresponding to a 1:1 molar ratio with radioactive 6,8-disulfooctanoic acid. These data indicated that the conjugate is an amide of 6,8-disulfooctanoic acid and L-lysine. The amount of radioactive 6,8-disulfooctanoic acid isolated from the hydrolyzed conjugate was inadequate to ascertain whether or not it was optically active. However, it is to be noted that the 6,8-disulfooctanoic acid was derived from natural lipoic acid, which is dextrorotatory.[20]

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(12) The molecular weights, calculated from sedimentation and diffusion constants by Dr. William R. Carroll of the Laboratory of Physical Biology, National Institute of Arthritis and Metabolic Diseases, are 4.8 million and 2.4 million, respectively. Complexes with similar properties were isolated previously from mammalian tissues (ref. 13, 14).

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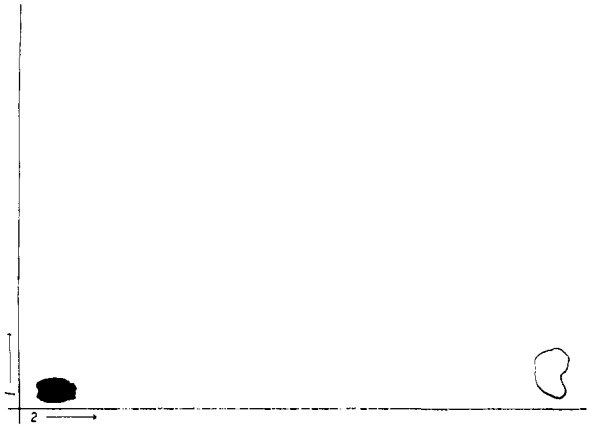
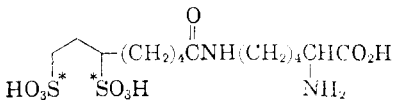


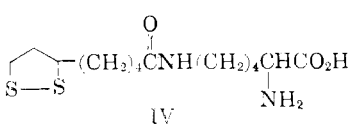
Fig. 2.—Radioautograph of two-dimensional paper chromatogram of hydrolysate of isolated conjugate. Solvent system 1 was butanol–acetic acid–water (4:1:5), system 2, water-saturated phenol–5% ammonia. The ninhydrin-positive spot was traced on the radioautograph.

Reaction of the conjugate with ninhydrin at room temperature indicated the presence of an α-amino group in the former compound. The conjugate reacted readily with 2,4-dinitrofluorobenzene[21] to give a yellow 2,4-dinitrophenyl (DNP) derivative. This derivative was hydrolyzed with 6 *N* hydrochloric acid and the hydrolysate was subjected to paper electrophoresis[22] together with samples of α-DNP-lysine and ϵ-DNP-lysine. The hydrolysate showed a single yellow spot which migrated at the same rate as α-DNP-lysine (Fig. 3). Neither substance reacted with ninhydrin at room temperature. ϵ-DNP-lysine, however, did react with ninhydrin under these conditions, due to the presence of an α-amino group in the former compound. It was concluded from these data that the isolated conjugate is ϵ-N-(6,8-disulfoöctanoyl)-L-lysine (III).



III

Confirmation of the structure assigned to the isolated conjugate was obtained by synthesis of ϵ-N-(DL-6,8-disulfoöctanoyl)-L-lysine and comparison of the synthetic and isolated compounds by paper electrophoresis and paper chromatography. A mixture of the two compounds migrated as a single spot under the conditions employed (Fig. 4). Furthermore, a mixture of the DNP derivatives of the two compounds migrated as a single spot (Fig. 5). ϵ-N-(DL-6,8-Disulfoöctanoyl)-L-lysine was prepared by oxidation of ϵ-N-(DL-lipoyl)-L-lysine (IV) with performic acid. ϵ-N-(DL-Lipoyl)-L-lysine was



IV

synthesized by reaction of DL-lipoic-isobutyl carbonic anhydride[8] with the copper complex of L-lysine.[23] Difficulty was experienced in isolation of the desired product due to a tendency of the lipoyl moiety to undergo polymerization.

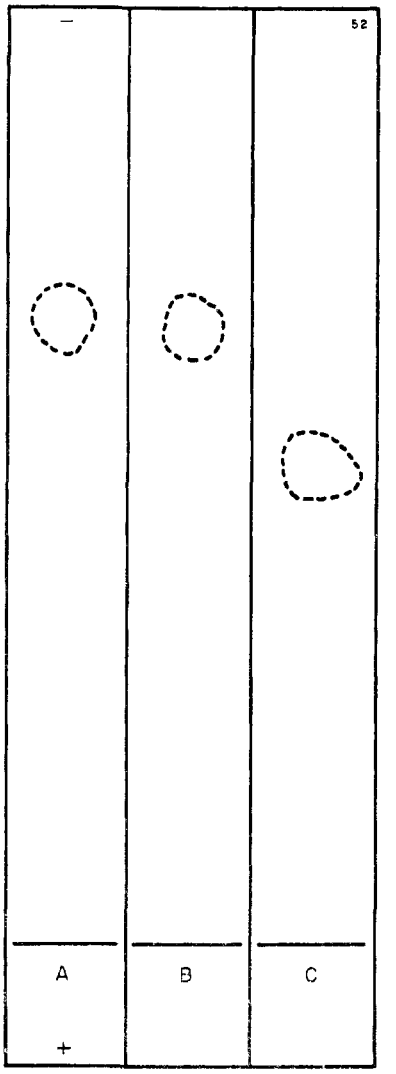


Fig. 3.—Comparative paper electrophoresis of hydrolysate of dinitrophenylated conjugate and DNP-lysine derivatives: A, 80 μg. of α-DNP-lysine; B, hydrolysate (calcd., 80 μg. of DNP-lysine); C, 80 μg. of ϵ-DNP-lysine hydrochloride. The electrolyte was 0.02 *M* sodium borate buffer, *p*H 8.9; 450 volts applied for 10 hr. The dashed lines outline yellow spots.

Due to paucity of highly purified radioactive pyruvate and α-ketoglutarate dehydrogenation complexes, the isolations described above were carried out with a protein preparation containing both complexes, in amounts comprising approximately 50 and 33%, respectively, of the total protein. Since 6,8-disulfoöctanoic acid and ϵ-N-(6,8-disulfoöctanoyl)-L-lysine were the major radioactive components found in the partial hydrolysate of the oxidized protein, it appeared that the lipoyl moiety was linked in both complexes to the ϵ-amino group of a lysine residue. Direct evidence supporting this conclusion was obtained by subjecting a preparation of each complex to the same procedure used

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(22) I. M. Lockhart and E. P. Abraham, *ibid.*, **62**, 645 (1956).
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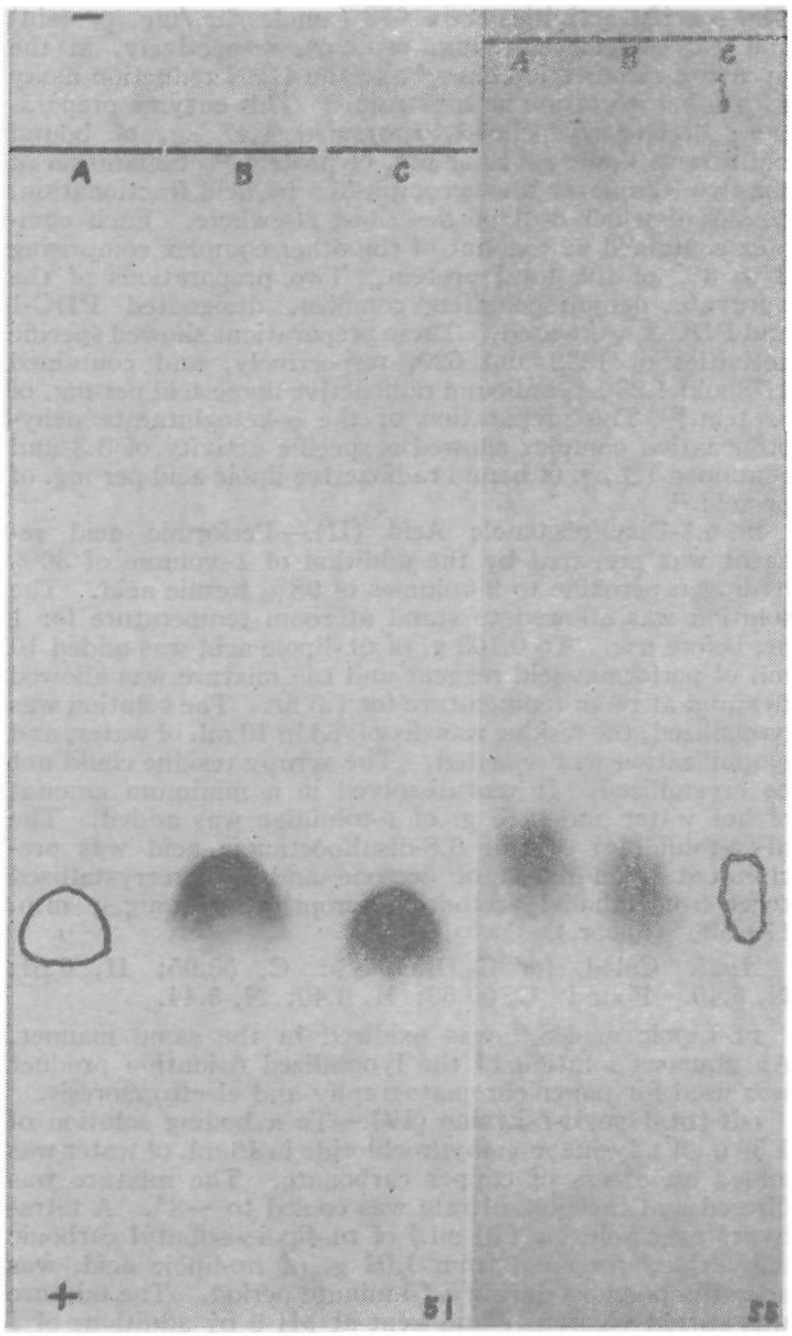

Fig. 4.—Comparative paper electrophoresis and paper chromatography of isolated and synthetic conjugates. Electrophoretic pattern (No. 51): A, 8.6 μg. of synthetic ϵ-N-(DL-6,8-disulfoöctanoyl)-L-lysine; B, mixture of A and C; C, 6.3 μg. (calcd.) of isolated conjugate. The electrolyte was 1 *N* acetic acid, *p*H 2.3; 400 volts applied for 3 hr. Chromatographic pattern (No. 55): samples as in No. 51, but in reverse order. The chromatogram was developed with butanol–acetic acid–water (2:1:1) for 54 hr.

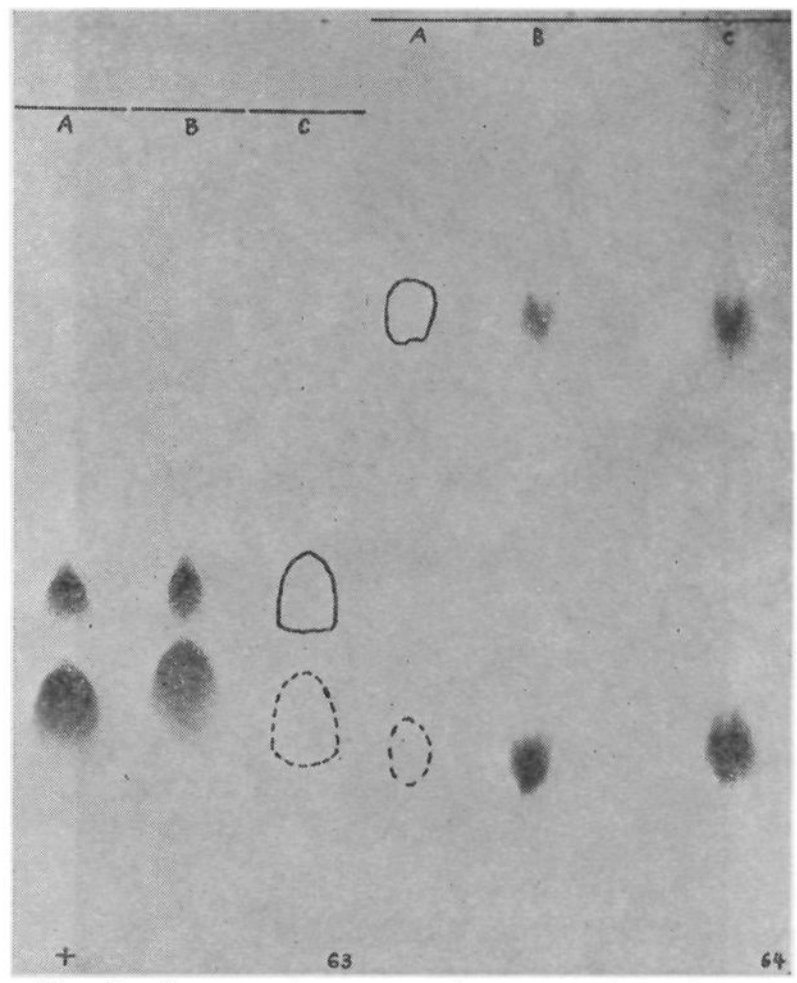

Fig. 5.—Comparative paper electrophoresis and paper chromatography of DNP derivatives of isolated and synthetic conjugates. Electrophoretic pattern (No. 63): A, 32 μg. (calcd.) of DNP derivative prepared from isolated conjugate; B, mixture of A and 30 μg. (calcd.) of DNP derivative prepared from synthetic ϵ-N-(DL-6,8-disulfoöctanoyl)-L-lysine; C, mixture of 20 μg. of synthetic conjugate (solid line) and 30 μg. (calcd.) of DNP derivative (dashed line). The electrolyte was 1 *N* acetic acid, *p*H 2.3; 400 volts applied for 2.5 hr. Chromatographic pattern (No. 64): samples as in No. 63, but in reverse order. The chromatogram was developed with butanol–acetic acid–water (2:1:1) for 18 hr.

with the mixture of the two complexes. From the partial hydrolysate of each oxidized complex was isolated a ninhydrin-positive, radioactive substance which migrated at the same rate as synthetic ϵ-N-(DL-6,8-disulfoöctanoyl)-L-lysine during paper electrophoresis and paper chromatography (*cf.* Fig. 4). The recovery of radioactivity as the conjugate amounted to 62 and 23%, respectively. The only other major radioactive substance found in the partial hydrolysates appeared to be 6,8-disulfoöctanoic acid.

Evidence was obtained which indicates that the lysine residue to which the lipoyl moiety is bound in the pyruvate and α-ketoglutarate dehydrogenation complexes is not N-terminal. An oxidized mixture of the two complexes was treated with 2,4-dinitrofluorobenzene prior to acid hydrolysis. Radioactive ϵ-N-(6,8-disulfoöctanoyl)-α-N-DNP-L-lysine, which would be expected if the lipoyl moiety were bound to an N-terminal lysine residue, was not found in the hydrolysate. However, a ninhydrin-positive, radioactive substance which migrated at the same rate as synthetic ϵ-N-(DL-6,8-disulfooctanoyl)-L-lysine (*cf.* Fig. 4) was isolated in an amount which corresponded to 67% of the original radioactivity. The only other major radioactive substance found in the hydrolysate appeared to be 6,8-disulfoöctanoic acid.

That the lysine residue to which the lipoyl moiety is bound in the pyruvate dehydrogenation complex is not C-terminal, and the nature of the amino acids about the lysine residue was indicated by analysis of three radioactive 6,8-disulfoöctanoyl peptides isolated from an acid hydrolysate of the oxidized complex (Fig. 6). The oxidized protein was hydrolyzed under milder conditions (12 *N* hydrochloric acid at 37° for 7 days) than those used previously. Qualitative amino acid analysis of the radioactive peptides utilizing two-dimensional paper chromatography indicated that peptide 1 contained lysine and aspartic acid, peptide 2 contained lysine, aspartic acid and alanine, and peptide 3 contained lysine and alanine. The data indicate that the sequence about the lysine residue is either Ala.Lys.Asp. or Asp.Lys.Ala. There was insufficient material for further characterization.

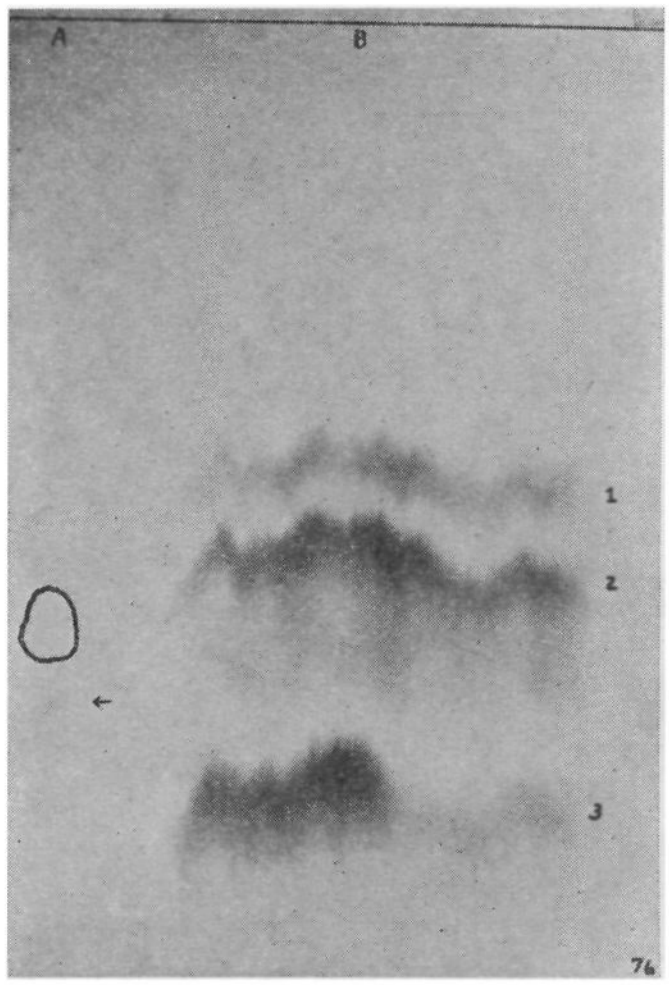

Fig. 6.—Radioautograph of one-dimensional paper chromatogram of 6,8-disulfoöctanoylpeptides: A, mixture of 22 μg. of synthetic ε-N-(DL-6,8-disulfoöctanoyl)-L-lysine (solid line) and 3 μg. of DL-6,8-disulfoöctanoic acid-S_2^{35} (arrow); B, purified fraction from acid hydrolysate (37°). The chromatogram was developed with butanol–acetic acid–water (2:1:1) for 56 hr.

Experimental

Methods.—Radioactivity was measured with a Tracerlab SC-16 windowless flow counter.[7] Electrophoresis was carried out on Whatman No. 3MM paper strips (3.0 × 30.6 cm.) in a Spinco model R apparatus at room temperature. The electrolyte used with the sulfonic acids was 1 *N* acetic acid, *p*H 2.3.[18] A potential difference of 400 volts was applied for the indicated time intervals. One-dimensional paper chromatography of the sulfonic acids was carried out by the descending method on Whatman No. 3MM paper with butanol–acetic acid–water (2:1:1). The solvent was allowed to drip from the bottom of the paper during development. For two-dimensional chromatography (descending method) Whatman No. 1 paper was used. The chromatograms were equilibrated with vapors from the aqueous phase in a closed cabinet for 12 to 24 hr. before development. Ninhydrin-reactive substances were located by spraying the air-dried papers with a solution of 0.2% ninhydrin in water-saturated butanol and allowing the color to develop at room temperature. In the preparation of radioautographs the papers were placed on Kodak No-Screen X-ray film for 3 to 5 days. Radioactive substances were eluted from the chromatograms with water in an apparatus similar to that of Kemble and Macpherson.[24]

Enzyme Preparations.—*E. coli* (Crookes strain) was grown and cell-free extracts were prepared essentially as described by Hager.[25] The growth medium contained DL-lipoic acid-S_2^{35} (2.12×10^4 c.p.m. per μg.)[26] at a level of 146 μg. per l. The cell extract was fractionated with protamine sulfate as described previously.[7] The eluate of the protamine precipitate was centrifuged for 2 hr. at 144,000 × *g* in the No. 40 rotor of a Spinco model L ultracentrifuge. The pellet contained both the pyruvate and α-ketoglutarate dehydrogenation complexes in amounts representing approximately 50 and 33%, respectively, of the total protein. The specific activities were 678 (μmoles/hr./mg. protein) and 1.0 (μmoles/min./mg. protein), respectively, in the pyruvate dismutation assay[7] and the DPN reduction assay with α-ketoglutarate as substrate.[25] This enzyme preparation, designated pellet-1, contained 1.47 μg. of bound radioactive lipoic acid per mg. of protein.[27] Separation of the two complexes was accomplished by acid fractionation, details of which will be described elsewhere. Each complex contained an amount of the other complex comprising 2 to 3% of the total protein. Two preparations of the pyruvate dehydrogenation complex, designated PDC-1 and PDC-2, were used. These preparations showed specific activities of 1332 and 620, respectively, and contained 1.78 and 1.23 μg. of bound radioactive lipoic acid per mg. of protein.[27] The preparation of the α-ketoglutarate dehydrogenation complex showed a specific activity of 3.3 and contained 1.1 μg. of bound radioactive lipoic acid per mg. of protein.[27]

DL-6,8-Disulfoöctanoic Acid (II).—Performic acid reagent was prepared by the addition of 1 volume of 30% hydrogen peroxide to 9 volumes of 98% formic acid. The solution was allowed to stand at room temperature for 1 hr. before use. To 0.103 g. of DL-lipoic acid was added 10 ml. of performic acid reagent and the mixture was allowed to stand at room temperature for 1.5 hr. The solution was lyophilized, the residue was dissolved in 10 ml. of water, and lyophilization was repeated. The syrupy residue could not be crystallized. It was dissolved in a minimum amount of hot water and 0.16 g. of *p*-toluidine was added. The di-(*p*-toluidine) salt of 6,8-disulfoöctanoic acid was precipitated by addition of acetone and was recrystallized twice from ethanol–acetone; microprisms (50 mg.); m.p. 179–180° (uncor.).

Anal. Calcd. for $C_{22}H_{34}O_8N_2S_2$: C, 50.95; H, 6.61; N, 5.40. Found: C, 50.53; H, 6.40; N, 5.44.

DL-Lipoic acid-S_2^{35} was oxidized in the same manner. *An aqueous solution of the lyophilized oxidation product* was used for paper chromatography and electrophoresis.

ε-N-(DL-Lipoyl)-L-Lysine (IV).—To a boiling solution of 1.36 g. of L-lysine monohydrochloride in 16 ml. of water was added an excess of copper carbonate. The mixture was filtered and the blue filtrate was cooled to −3°. A tetrahydrofuran solution (10 ml.) of DL-lipoic-isobutyl carbonic anhydride,[8] prepared from 1.03 g. of DL-lipoic acid, was added in portions during a 15-minute period. The mixture was stirred vigorously and kept at *p*H 9 by additions of 1 *N* sodium hydroxide solution. Stirring was continued for approximately 30 minutes, while the mixture was allowed to warm to room temperature. The mixture was filtered and the pale blue solid was washed with several portions of water. The filtrate and wash fluid were discarded. The solid was dissolved in 25 ml. of 1 *N* sulfuric acid and hydrogen sulfide was passed into the solution for approximately 15 minutes. The mixture was filtered and the dark brown filtrate was diluted with water to 100 ml. The solution was passed through a column prepared from 10 g. of Dowex 50-X2 (50 to 100 mesh) in the hydrogen cycle. The column was washed with water until the effluent was colorless and then was eluted with 1 *N* ammonium hydroxide solution. The ninhydrin-positive fraction was lyophilized and the solid was washed consecutively with 5 ml. of cold water, 1 ml. of ethanol and 1 ml. of ether and then dried *in vacuo;* weight 461 mg. (28% yield); $\lambda_{max}^{0.1\ N\ NaOH}$ 330 mμ (ε 104). The product was treated as described below to obtain a pale yellow amorphous solid; ε 116 at 330 mμ; m.p. 225–229° dec.; R_f = 0.60 in butanol–acetic acid–water (4:1:5).

Anal. Calcd. for $C_{14}H_{26}N_2O_3S_2$: C, 50.27; H, 7.83; N, 8.37. Found: C, 50.21; H, 8.10; N, 8.33.

The crude product may contain variable amounts of linear disulfide polymer of ε-N-(DL-lipoyl)-L-lysine, as indicated by a low extinction coefficient and by paper chromatography (R_f of polymer is 0.0). In such cases the product was

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(27) The lipoic acid content (calculated as (+)-lipoic acid) of these preparations determined by manometric assay (ref. 28) of samples hydrolyzed by autoclaving with 0.1 *N* sodium hydroxide under nitrogen and in the presence of 0.2% crystalline bovine serum albumin (ref. 29) was 75 to 80% of that based on radioactivity.

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treated as described below to depolymerize the polymer[16] and recover the monomer. A 150-mg. sample of crude product, ϵ 66 at 330 mμ, was dissolved in 3 ml. of 0.5 *N* aqueous sodium hydroxide and the solution was heated on a steam-bath until the absorbance at 330 mμ showed no further increase (approximately 15 minutes). The solution was filtered to remove a small amount of black precipitate, presumably copper sulfide. The filtrate was diluted with cold water to 40 ml. and passed through a column prepared from 4 g. of Amberlite IRC-50 in the hydrogen cycle. The column was washed with 50 ml. of cold water. The effluent and wash fluid were combined and lyophilized to obtain 65 mg. of a pale yellow solid, $\lambda_{max}^{0.1\ N\ NaOH}$ 330 mμ (ϵ 112). Alternatively, the original alkaline solution may be neutralized cautiously in the cold with 1 *N* acetic acid to recover ϵ-N-(DL-lipoyl)-L-lysine. However, this procedure occasionally resulted in formation of polymer, as indicated by the low extinction coefficient of the precipitate.

ϵ-N-(DL-6,8-Disulfoöctanoyl)-L-Lysine (III).—ϵ-N-(DL-Lipoyl)-L-lysine was oxidized with performic acid as described above. The product was a viscous oil which chromatographed as a single spot (*cf.* Fig. 4). It yielded a solid *p*-toluidine salt which, however, was too hygroscopic to obtain a m.p.

Isolation of Radioactive Conjugate from Partial Hydrolysate of Oxidized Pellet.—A 176-mg. sample of pellet-1 was oxidized with 6 ml. of performic acid reagent at 4° for 16 hr. The clear solution was lyophilized, the residue was dissolved in 10 ml. of water, and lyophilization was repeated. The light yellow residue was dissolved in 12 ml. of 12 *N* hydrochloric acid and the solution was heated in a sealed tube under nitrogen for 3 hr. at 105°. The hydrolysate was evaporated to dryness *in vacuo* over solid potassium hydroxide and calcium chloride. The residue was dissolved in 10 ml. of water and the solution was extracted with 10-ml. portions of chloroform and ethyl acetate to remove non-radioactive, yellow, fluorescent material. Recovery of radioactivity at this stage was 84%. The aqueous layer was lyophilized and the residue was dissolved in 2 ml. of water and applied to a column (16 ml.) of Dowex 50W-X8 (200 to 400 mesh) in the hydrogen cycle. Water was passed through the column and radioactive material began to emerge after 6 ml. of effluent was collected. The recovery of radioactivity in the next 10 ml. of effluent was essentially quantitative. The solution was lyophilized, the residue was dissolved in 0.7 ml. of water and 0.1-ml. aliquots were applied in a thin line 5 cm. from the ends (cathode ends) of seven filter paper strips. Electrophoresis was carried out as described above for 3 hr. A radioautograph of the paper strips showed spreading of the radioactive material up to the anode end, with concentration in a section 2.5 to 15.5 cm. from the origin. This section was cut from each of the strips and the adsorbed material was eluted with water. The recovery of radioactivity in the combined eluates was 51% (based on original radioactivity). Further purification was achieved by one-dimensional paper chromatography with the butanol–acetic acid–water system for 53 hr. The major radioactive band, located by radioautography, was cut out and the adsorbed material was eluted with water. Aliquots were subjected to paper electrophoresis and paper chromatography under the conditions described above. A single ninhydrin-positive, radioactive spot was detected, indicating that the isolated conjugate was homogenous. Recovery of radioactivity in the conjugate amounted to 47% of the original radioactivity. In a second isolation, electrophoresis was carried out for 1.75 hr. Sections of the paper strips 0.3 to 2.3 cm., 2.3 to 7.5 cm., and 7.5 to 15.2 cm. from the origin were cut out and the adsorbed material was eluted (recovery of radioactivity, 92%) and subjected to paper chromatography. The conjugate, present in the first and second sections of the paper strips, was recovered from the paper chromatogram in 60% yield (based on original radioactivity). A ninhydrin-negative, radioactive substance which migrated at the same rate as authentic DL-6,8-disulfoöctanoic acid-S_2^{35}, was present in the second and third sections of the paper strips and was recovered from the paper chromatogram in 20% yield.

Characterization of Isolated Conjugate. (a) L-Lysine and Radioactive 6,8-Disulfoöctanoic Acid from Conjugate.—A sample of the isolated conjugate, equivalent to 160 μg. of bound 6,8-disulfoöctanoic acid, was heated with 1 ml. of 6 *N* hydrochloric acid in a sealed tube under nitrogen for 18 hr. at 105°. The hydrolysate was evaporated to dryness and the residue was dissolved in 0.2 ml. of water. A 0.04-ml. aliquot was subjected to two-dimensional paper chromatography with butanol–acetic acid–water (4:1:5)[30] in the first dimension (16 hr.) and water-saturated phenol–5% ammonia in the second dimension (20 hr.). A radioactive spot and, in addition, a ninhydrin-positive spot were detected on the chromatogram (Fig. 2). Comparison with a two-dimensional chromatogram prepared with known amino acids under the same conditions indicated that the ninhydrin-positive spot was lysine. When an aliquot of the hydrolysate was co-chromatographed with authentic L-lysine monohydrochloride in the phenol–ammonia system, a single ninhydrin-positive spot was detected on the chromatogram. The presence of lysine in the hydrolysate was indicated also by two-dimensional chromatography according to Levy and Chung[31] with butanol–acetic acid–water (4:1:5) and *m*-cresol–phenol (2:1)–borate buffer (*p*H 8.3) as the solvent systems. However, under these conditions the lysine spot from both the hydrolysate and a synthetic mixture of L-lysine monohydrochloride and 6,8-disulfoöctanoic acid showed appreciable tailing and a lower R_f than that indicated by Levy and Chung. This phenomenon can be attributed apparently to the strongly acidic nature of the samples (*cf.* ref. 32).

The radioactive spot near the origin of the chromatogram (Fig. 2) was cut out and the adsorbed material was eluted with water (recovery of radioactivity, 90%). When an aliquot of the eluate was co-chromatographed with authentic DL-6,8-disulfoöctanoic acid-S_2^{35} in butanol–acetic acid–water (2:1:1) a single radioactive spot was detected on the chromatogram.

Microbiological assay[33] of the hydrolysate with *Streptococcus faecalis* 8043 showed the presence of 74 μg. of L-lysine, which is 97% of the value expected if L-lysine and 6,8-disulfoöctanoic acid were present in a 1:1 molar ratio. No response in the microbiological assay was obtained with D-lysine.

(b) α-DNP-Lysine from Conjugate.—To a 112-μg. (calcd.) sample of the isolated conjugate was added a solution of 0.48 mg. of sodium bicarbonate in 0.75 ml. of water. A solution of 0.15 mg. of 2,4-dinitrofluorobenzene in 1.5 ml. of ethanol was added and the mixture was stirred for 3.5 hr. The solution was concentrated in a stream of nitrogen and extracted three times with 1-ml. portions of ether. The aqueous layer was evaporated to dryness and the residue was heated with 1 ml. of 6 *N* hydrochloric acid in a sealed tube under nitrogen for 18 hr. at 105°. The hydrolysate was evaporated to dryness, the residue was dissolved in 0.5 ml. of water and the solution was extracted with two 1-ml. portions of ether. No color was detected in the ether extracts. The yellow aqueous solution was concentrated and then subjected to paper electrophoresis[22] together with samples of authentic α-DNP-lysine[34] and ϵ-DNP-lysine hydrochloride.[35] The electrophoretic pattern is shown in Fig. 3. When the paper strips were sprayed with ninhydrin solution, the spot corresponding to ϵ-DNP-lysine developed a purple color, whereas the other two spots remained yellow.

(c) Comparison of Isolated and Synthetic Conjugates.—The isolated conjugate, synthetic ϵ-N-(DL-6,8-disulfoöctanoyl)-L-lysine, and a mixture of the two compounds were subjected to paper electrophoresis and paper chromatography. The patterns are shown in Fig. 4. The radioactive spots coincided with the ninhydrin-positive spots.

A 177-μg. (calcd.) sample of the isolated conjugate and a 2.2-mg. sample of synthetic ϵ-N-(DL-6,8-disulfoöctanoyl)-L-lysine were treated with 2,4-dinitrofluorobenzene essentially as described above. Each of the ether-extracted aqueous solutions was passed through a column of Dowex 50W-X8 in the hydrogen cycle and the columns were washed with water. Recovery of radioactivity was 92%. The yellow effluents were lyophilized and the residues were subjected to paper electrophoresis and paper chromatography. The patterns are shown in Fig. 5. The major radioactive spot, which was yellow and did not react with ninhydrin, is ϵ-N-(6,8-disulfoöctanoyl-S_2^{35})-α-N-DNP-L-ly-

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sine. The minor radioactive spot was colorless and reacted with ninhydrin. It represents a small amount of the isolated conjugate which did not undergo dinitrophenylation.

Isolation of Radioactive Conjugate from Partial Hydrolysates of Oxidized Pyruvate and α-Ketoglutarate Dehydrogenation Complexes.—A preparation of the pyruvate dehydrogenation complex (PDC-1, 54 mg. of protein) was oxidized with performic acid, then hydrolyzed with 12 *N* hydrochloric acid, and the hydrolysate was processed essentially as described above. A ninhydrin-positive, radioactive conjugate was isolated in an amount which represented 62% of the original radioactivity. A preparation of the α-ketoglutarate dehydrogenation complex (46.8 mg. of protein) was treated in a similar manner. The yield of ninhydrin-positive, radioactive conjugate was 23%, based on the original radioactivity. The isolated conjugates and synthetic ε-N-(DL-6,8-disulfoöctanoyl)-L-lysine were compared separately and as mixtures using paper electrophoresis and paper chromatography (*cf.* Fig. 4). The migration rates of the three substances were identical.

The only other major radioactive substance found in the partial hydrolysates migrated at the same rate in butanol–acetic acid–water, both before and after hydrolysis with 6 *N* hydrochloric acid, as authentic DL-6,8-disulfoöctanoic acid-S_2^{35}. Also, it did not react with ninhydrin, nor did it yield ninhydrin-reactive material when hydrolyzed.

Isolation of Radioactive Conjugate from Partial Hydrolysate of Oxidized Dinitrophenylated Pellet.—An 80-mg. sample of pellet-1 was oxidized with performic acid as described above. The oxidized protein was suspended in a solution of 0.2 g. of sodium bicarbonate in 2 ml. of water. A solution of 0.2 ml. of 2,4-dinitrofluorobenzene in 4 ml. of ethanol was added and the mixture was stirred for 4 hr. The precipitate was collected by centrifugation and washed consecutively with 25 ml. of water, 10 ml. of ethanol and 15 ml. of ether. No radioactivity was found in the wash fluid. The dried precipitate was hydrolyzed with 12 *N* hydrochloric acid as described above. The hydrolysate was evaporated to dryness, the residue was dissolved in 1 ml. of water and the solution was applied to a column of Dowex 50W-X8 in the hydrogen cycle. When the column was washed with water, 83% of the original radioactivity was recovered in the effluent. The radioactive fractions were colorless, indicating the absence of DNP derivatives. Elution of the column with 1 and 6 *N* hydrochloric acid gave intensely yellow solutions, which were not radioactive, and were discarded. The water effluent and samples of synthetic ε-N-(DL-6,8-disulfoöctanoyl)-L-lysine, the DNP derivative prepared from the latter substance, and DL-6,8-disulfoöctanoic acid-S_2^{35} were chromatographed on paper. A radioautograph of the chromatogram showed a major radioactive band and a faster-moving diffuse band in areas corresponding to ε-N-(DL-6,8-disulfoöctanoyl)-L-lysine and DL-6,8-disulfoöctanoic acid-S_2^{35}, respectively. No radioactivity was observed in the area corresponding to ε-N-(DL-6,8-disulfoöctanoyl)-α-N-DNP-L-lysine. The major radioactive band was cut out and the adsorbed material was eluted and rechromatographed on paper. Recovery of ninhydrin-positive, radioactive conjugate from the second chromatogram was 67%, based on the original radioactivity. Comparison of the isolated conjugate and synthetic ε-N-(DL-6,8-disulfoöctanoyl)-L-lysine using paper electrophoresis and paper chromatography (*cf.* Fig. 4) showed that their migration rates were identical.

Isolation and Composition of Radioactive 6,8-Disulfooctanoylpeptides from Partial Hydrolysate of Oxidized Pyruvate Dehydrogenation Complex.—A 150-mg. sample of the pyruvate dehydrogenation complex (PDC-2) was oxidized with performic acid. The oxidized protein was dissolved in 11 ml. of 12 *N* hydrochloric acid and the solution was allowed to stand in a sealed tube under nitrogen for 7 days at 37°. The partial hydrolysate was fractionated by passage through Dowex 50W-X8, followed by paper electrophoresis and then paper chromatography, essentially as described above. Recovery of radioactivity from the paper chromatogram was 68%. The radioactive material was rechromatographed on paper. A radioautograph of the chromatogram showed three radioactive bands with different mobilities than synthetic ε-N-(DL-6,8-disulfooctanoyl)-L-lysine and DL-6,8-disulfoöctanoic acid-S_2^{35} (Fig. 6). The radioactive bands were cut out and the adsorbed material was eluted with water. Recovery of radioactivity in bands 1, 2 and 3 was 9, 19 and 18%, respectively. The eluate of band 2 contained a small amount (*ca.* 5%) of radioactive ε-N-(6,8-disulfoöctanoyl)-L-lysine as indicated by paper electrophoresis and paper chromatography (conditions as in Fig. 4). The eluates were evaporated to dryness and each residue was hydrolyzed with 6 *N* hydrochloric acid in a sealed tube for 15 hr. at 105°. The hydrolysates were subjected to two-dimensional paper chromatography under conditions as in Fig. 2. Comparison with a chromatogram prepared under the same conditions with known amino acids indicated that the hydrolysate of band 1 contained lysine and aspartic acid, the hydrolysate of band 2 contained lysine, aspartic acid and alanine, and the hydrolysate of band 3 contained lysine and alanine. Visual estimation indicated that the amino acids were present in approximately equal amounts. On each chromatogram, a single radioactive spot, presumably 6,8-disulfoöctanoic acid, was detected near the origin (*cf.* Fig. 2).

Enzymatic Reduction of ε-N-(DL-Lipoyl)-L-Lysine.—Dihydrolipoic dehydrogenase (specific activity, 4500 μmoles/hr./mg. protein in pyruvate dismutation assay at *p*H 7.0)[7,35] was prepared from *E. coli*.[37] Reduction of synthetic substrates by DPNH was carried out in a reaction mixture containing 50 μmoles of potassium phosphate buffer, *p*H 8.4 (*p*H optimum), 1 to 3 μmoles of substrate, 0.1 μmole of DPNH and 2 to 4 μg. of enzyme in a final volume of 1.0 ml. The decrease in absorbance at 340 mμ was recorded with a Beckman DK-2 spectrophotometer. The rate of reduction of ε-N-(DL-lipoyl)-L-lysine was 2 to 3 times as fast as the rate of reduction of DL-lipoamide.[8] The relative rates with 3 μg. of enzyme and 3 μmoles of substrate were 38 and 13 μmoles/min./mg. protein, respectively. These are not maximum rates, however, since an apparent inhibition by DPNH was observed (*cf.* ref. 38). DL-Lipoic acid was not reduced at a measurable rate under the conditions used.

Discussion

These studies and previous work[8] demonstrate that protein-bound lipoic acid is the functional form of lipoic acid and that it is non-dissociable. In view of these results, the earlier proposal of Reed and DeBusk[39] that "lipothiamide pyrophosphate" is the functional form of lipoic acid is untenable. No satisfactory explanation of their data is apparent at the present time. The only known method of releasing protein-bound lipoic acid, without denaturing protein, is by incubation with the enzyme, lipoyl-X hydrolase.[8,40] This enzyme presumably cleaves the covalent bond between lipoic acid and the lysine residue. Reactivation of the apoenzyme, which presumably involves reforming this bond, requires incubation with lipoic acid, ATP, and a lipoic acid-activating enzyme system.[7] It is not yet possible to specify which enzymatic component of the pyruvate and α-ketoglutarate dehydrogenation complexes contains the bound lipoic acid. However, previous work[8] indicates that the lipoyl moiety is not attached to either dihydrolipoic transacetylase or dihydrolipoic dehydrogenase.

Elucidation of the nature of protein-bound lipoic acid provides a rational explanation of the observations that model reactions catalyzed by the α-keto acid dehydrogenation complexes and enzymatic components thereof proceed at faster rates with lipoamide or dihydrolipoamide than with lipoic acid or dihydrolipoic acid.[8,41–43] The amides bear a

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closer structural resemblance to the natural substrate than do the acids. Consistent with this interpretation is the present observation that the rate of reduction of ε-N-(DL-lipoyl)-L-lysine by DPNH in the presence of *E. coli* dihydrolipoic dehydrogenase was 2 to 3 times as fast as the rate of reduction of DL-lipoamide.

It is to be noted that ε-N-lipoyl-L-lysine bears a striking structural resemblance to biocytin (ε-N-biotinyl-L-lysine) which was isolated previously from yeast autolysate.[44] Biotin is known to occur naturally in association with proteins[45] and protein-bound biotin has been reported to be involved in the synthesis of fatty acids from acetate.[46] Although the nature of the moiety to which biotin is bound has not been established, it seems highly probably that it is bound to the ε-amino group of a lysine residue. It would appear that comparative studies of the release of protein-bound biotin and the incorporation of biotin into protein are in order.

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AUSTIN, TEXAS

[CONTRIBUTION FROM THE ST. LOUIS RESEARCH DEPARTMENT, ORGANIC CHEMICALS DIVISION, MONSANTO CHEMICAL CO.]

Reactions of Phosphorus Compounds. II. A New Type of Oxidizing Agent—Trichloroacetamides

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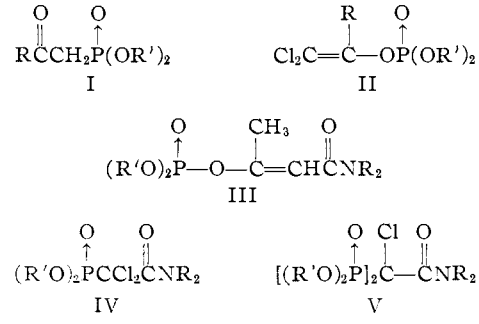
Phosphites and phosphines react with N,N-disubstituted trichloroacetamides to give trichlorovinylamines (VIII) in 23–83% yield and the corresponding quadruply-connected phosphorus compound. This new reaction which appears to be general for trialkyl phosphites and tertiary phosphines entails the oxidation of phosphorus by an amide with concomitant migration of a chlorine atom. This reaction also represents a novel method for the preparation of a new class of vinylamines. The mechanism of the reaction leading to trichlorovinylamines may involve the same type of intermediate as that proposed in the Perkow rearrangement. The unique differences in these two reactions are discussed.

The reaction of 2-monohaloacetamides[1] and 2-monohaloacetates[1,2] with trialkyl phosphites has been shown to give phosphonates (I) (Michaelis–Arbuzov reaction[3]) and not vinyl phosphates (II Cl = H) (Perkow rearrangement[4]). Whetstone and Stiles,[5] however, have isolated vinyl phosphates (III) from the reaction of 2-chloro-N,N-dialkylacetoacetamides and trialkyl phosphites. Vinyl phosphates[2,4,6,7] (II, R = hydrogen, alkyl, aryl, alkoxyl) were also formed from the reaction of trialkyl phosphites with trihaloesters, aldehydes and ketones.

Allen and Johnson[7a,c] have reported that trichloroacetamides gave vinyl phosphates [II, R = $N(C_2H_5)_2$] in a manner analogous to trihaloesters and aldehydes. Because of the similarity of the reactions of esters and amides, one would have expected these haloamides to yield vinyl phosphates.



We have found, however, that the reaction of trialkyl phosphites and α-trihaloacetamides yielded trichlorovinylamines (VIII). Attempts to isolate phosphates [II, R = $N(alkyl)_2$ or $N(aryl)_2$] and phosphonates IV and V were unsuccessful. These by-products may have been formed since varying amounts of alkyl chlorides were obtained in some of the reactions studied. The trialkyl phosphites were converted to trialkyl phosphates. Unlike the Perkow and Arbuzov reactions, this new rearrangement is not limited to phosphorous esters. Vinylamines (VIII) were also produced by the action of tertiary phosphines on trichloroacetamides.

The over-all transformation entailed the oxidation of a triply-connected phosphorus compound with concomitant migration of a chlorine atom.

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