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

The Amino Acid Sequence Around the N^{ϵ}-Lipoyllysine Residue in α-Keto Acid Dehydrogenation Complexes[1]

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RECEIVED JULY 17, 1961

Highly purified preparations of the *Escherichia coli* pyruvate and α-ketoglutarate dehydrogenation complexes containing bound radioactive lipoic acid were oxidized with performic acid and then subjected to partial acid hydrolysis. The radioactive 6,8-disulfoöctanoylpeptides were isolated and identified. It is concluded that the pyruvate dehydrogenation complex contains the sequence Gly.Asp.ϵ-Lipoyl-Lys.Ala and that the α-ketoglutarate dehydrogenation complex contains the sequence Thr.Asp.ϵ-Lipoyl-Lys.Val.(Val,Leu).Glu. Conversion of the α-aspartyl peptides to the $\alpha\beta$ and β isomers during acid hydrolysis is reported.

In a previous publication from this Laboratory[3] it was reported that the lipoyl moiety which is a functional component of the CoA- and diphosphopyridine nucleotide (DPN)-linked pyruvate and α-ketoglutarate dehydrogenation complexes of *Escherichia coli* is bound in amide linkage to the ϵ-amino group of a lysine residue. It was of interest to determine the amino acid sequence around the N^{ϵ}-lipoyllysine residue in the two enzyme complexes, in the hope that this information might ultimately shed light on the chemical basis of the substrate specificity of the two complexes. Preliminary experiments[3] indicated that the sequence around the N^{ϵ}-lipoyllysine residue in the pyruvate dehydrogenation complex is either Ala.Lys.Asp. or Asp.Lys.Ala. The present paper describes some further experiments which indicate that the sequence Gly.Asp.ϵ-Lipoyl-Lys.Ala is present in the pyruvate dehydrogenation complex and the sequence Thr.Asp.ϵ-Lipoyl-Lys.Val.(Val,Leu).Glu is present in the α-ketoglutarate dehydrogenation complex.

Highly purified preparations of the complexes containing bound lipoic acid-S_2^{35} were oxidized with performic acid and then partially hydrolyzed with 12 *N* hydrochloric acid or 12 *N* hydrochloric acid-acetic acid (1:1) at 37°. The hydrolysates were passed through columns of Dowex 50W-X8 in the hydrogen cycle to remove the majority of nonradioactive peptides. Partial separation of the radioactive 6,8-disulfoöctanoyl (DSO) peptides was achieved by large scale paper chromatography with butanol–acetic acid–water (2:1:1) as the solvent system. Radioactive bands were located by means of radioautographs, eluted and purified further by paper electrophoresis in 1 *N* acetic acid, *p*H 2.3.[4] The electrophoretic patterns of the isolated radioactive peptides are shown in Figs. 1 and 2. The yields of the various peptides are given in Tables I and II. The total recoveries of S^{35} are low (42–54%) and result mainly from the fact that in cutting out the radioactive bands to be eluted, purity rather than high yields was desired. Samples of each peptide were taken for qualitative amino acid analysis utilizing two-dimensional paper chromatography and for amino end group determination by the 2,4-dinitrophenyl (DNP) technique.[5] Quantitative amino acid analysis was also performed on certain of the peptides. The results are shown in Tables III and IV.

TABLE I

RECOVERIES OF S^{35}-LABELED PEPTIDES FROM PARTIAL HYDROLYSATES OF OXIDIZED PYRUVATE DEHYDROGENATION COMPLEX

Band no.	Recovery of radioactive material, % Expt. 1	Expt. 2
P-1	8	..
P-2 (A + B)	4	5
P-2C	8	11
P-3A	5	8
P-3B	8	..
P-3C	5	..
P-4	16	11
P-5 (A + B)	..	14

TABLE II

RECOVERIES OF S^{35}-LABELED PEPTIDES FROM PARTIAL HYDROLYSATES OF OXIDIZED α-KETOGLUTARATE DEHYDROGENATION COMPLEX

Band no.	Recovery of radioactive material, % Expt. 1	Expt. 2
K-1	24	12
K-2	10	..
K-3	16	30

Difficulties were experienced in attempts to establish the structures of the radioactive peptides present in the hydrolysates of the pyruvate dehydrogenation complex. The mixture appeared more complex than would have been expected on the basis of a splitting of peptide and amide bonds during hydrolysis. For example, three peptides were isolated which appeared to have the structure Asp.-ϵ-DSO-Lys.Ala. Another three appeared to have the structure Asp.ϵ-DSO-Lys. Similar observations were made by Naughton, *et al.*,[6] in their investigation of P^{32}-labeled phosphopeptides produced by partial acid hydrolysis of diisopropoxyphosphinyl-P^{32} derivatives of chymotrypsin, trypsin and elastase, which contain the sequence Asp.Ser.Gly around the reactive serine residue. A satisfactory explanation of these results appears to be that interconversion of aspartyl residues, as represented below, occurs during acid hydrolysis.[6]

(1) This investigation was supported in part by a research grant (RG-6590(C1)) from the Division of General Medical Sciences, United States Public Health Service.

(2) Rosalie B. Hite Postdoctoral Fellow, 1959–1961.

(3) H. Nawa, W. T. Brady, M. Koike and L. J. Reed, *J. Am. Chem. Soc.*, **82**, 896 (1960).

(4) J. R. Kimmel, E. O. P. Thompson and E. L. Smith, *J. Biol. Chem.*, **217**, 151 (1955).

(5) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(6) M. A. Naughton, F. Sanger, B. S. Hartley and D. C. Shaw, *ibid.*, **77**, 149 (1960).

TABLE III

AMINO ACID COMPOSITION AND N-TERMINAL RESIDUES OF RADIOACTIVE PEPTIDES FROM PYRUVATE DEHYDROGENATION COMPLEX

Peptide no.	Amino acids present	Molar ratio	N-terminal residue	Probable structure
P-1	Asp,Lys	1.0:1.0	Asp	β-Asp.ε-DSO-Lys
P-2A	Asp,Lys		Asp	α-Asp.ε-DSO-Lys
P-2B	Gly,Asp,Lys		Gly	Gly.αβ-Asp.ε-DSO-Lys
P-2C	Asp,Lys,Ala	1.1:1.0:1.0[a]	Asp	β-Asp.ε-DSO-Lys.Ala
P-3A	Asp,Lys,Ala	1.00:0.93:0.87[b]	Asp	αβ-Asp.ε-DSO-Lys.Ala
P-3B	Asp,Lys			?
P-3C	Lys			ε-DSO-Lys
P-4	Lys,Ala	1.0:1.0	Lys	ε-DSO-Lys.Ala
P-5A	Gly,Asp,Lys,Ala	0.6:0.9:1.0:0.9	Gly	Gly.α-Asp.ε-DSO-Lys.Ala
P-5B	Asp,Lys,Ala	1.2:1.0:1.2	Asp	α-Asp.ε-DSO-Lys.Ala

[a] Analysis by means of a Spinco amino acid analyzer showed the following molar ratios: Asp, 0.93; Lys, 1.00; Ala, 0.87. [b] Analyzed by means of a Spinco amino acid analyzer.

Swallow and Abraham[7] had shown previously that N^{ϵ}-(α-aspartyl)-L-lysine and N^{ϵ}-(β-aspartyl)-L-lysine are interconvertible when heated in acid solution and that both isomers are converted

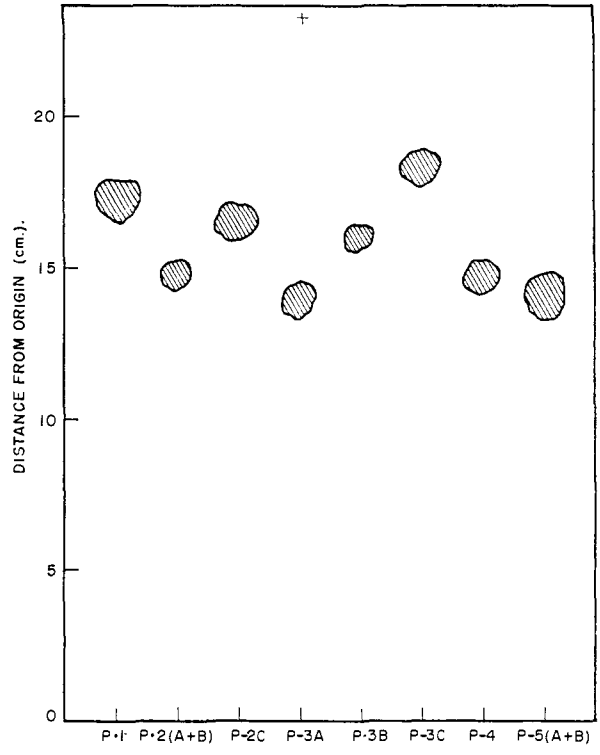
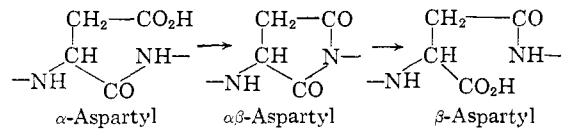


Fig. 1.—Tracing of radioautograph showing electrophoretic pattern of S^{35}-labeled 6,8-disulfoöctanoylpeptides isolated from partial hydrolysates of oxidized pyruvate dehydrogenation complex. The electrolyte was 1 *N* acetic acid, *p*H 2.3; 400 volts applied for 4 hr.

mainly into the αβ-aspartyl derivative. An αβ-aspartyl derivative is formed also from N^{α}-(α-aspartyl)-L-lysine but is not as stable as the N^{ϵ}-(αβ-aspartyl)-L-lysine.[7]



(7) D. L. Swallow and E. P. Abraham, *Biochem. J.*, **70**, 364 (1958).

Two of the radioactive compounds isolated from partial hydrolysates of the oxidized pyruvate dehydrogenation complex were readily identifiable. Compound P-3C gave radioactive 6,8-disulfooctanoic acid and lysine when hydrolyzed and

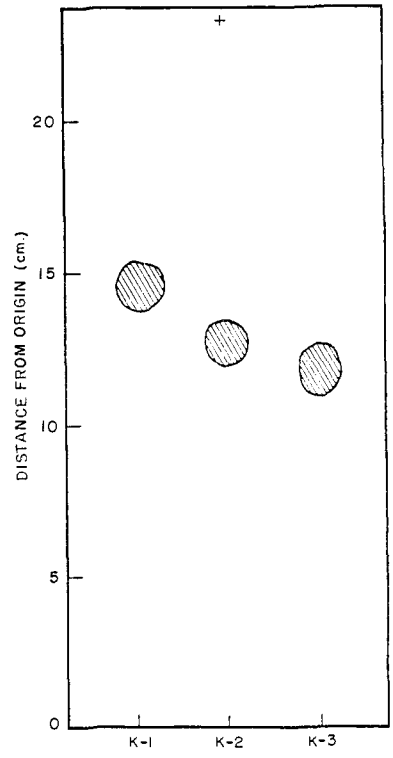


Fig. 2.—Tracing of radioautograph showing electrophoretic pattern of S^{35}-labeled 6,8-disulfoöctanoylpeptides isolated from partial hydrolysates of oxidized α-ketoglutarate dehydrogenation complex. Conditions were as given in legend of Fig. 1.

showed the same migration rates as synthetic N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine.[3] Peptide P-4 gave equimolar amounts of lysine and alanine on complete hydrolysis and α-DNP-lysine by amino end group analysis. It therefore has the structure N^{ϵ}-(6,8-disulfoöctanoyl)-lysylalanine. Peptides P-1 and P-2C gave a blue color with ninhydrin, indicating that these are β-aspartyl peptides. Bryant,

TABLE IV

AMINO ACID COMPOSITION AND N-TERMINAL RESIDUES OF RADIOACTIVE PEPTIDES FROM α-KETOGLUTARATE DEHYDROGENATION COMPLEX

Peptide no.	Amino acids present	Molar ratio	N-terminal residue	Probable structure
K-1	Thr,Asp,Lys	1.0:1.1:1.0[a]	Thr	Thr.αβ-Asp.ε-DSO-Lys
K-2	Lys,Val,Leu		Lys	ε-DSO-Lys.Val.(Val,Leu)
K-3	Thr,Asp,Lys,Val,Leu,Glu	1.2:1.1:1.0:2.0:1.4:0.8	Thr	Thr.Asp.ε-DSO-Lys.Val.(Val,Leu).Glu

[a] Analysis by means of a Spinco amino acid analyzer showed the following molar ratios: Thr, 0.98; Asp, 1.08; Lys, 1.00.

et al.,[8] reported that β-aspartyl peptides give a blue color when paper chromatograms are treated with ninhydrin and dried rapidly at 100–120°, whereas α-aspartyl peptides give a purple color. This distinction has been confirmed by other workers (*cf.*

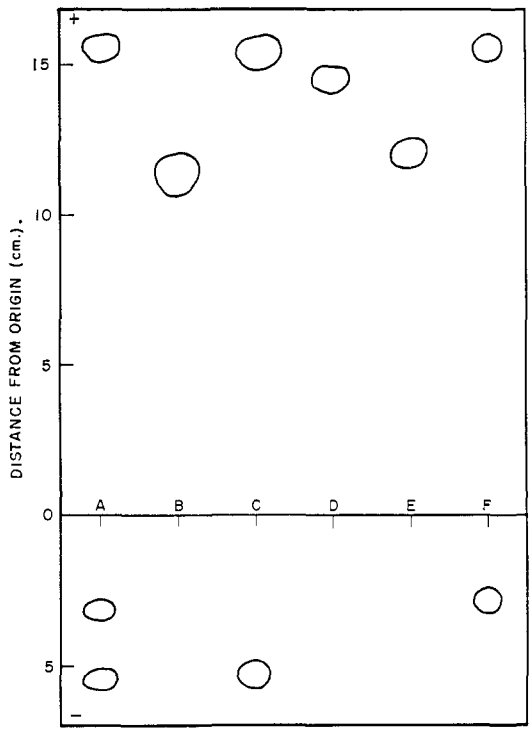


Fig. 3.—Electrophoretic pattern of synthetic 6,8-disulfooctanoylpeptides before and after treatment with leucine aminopeptidase (LAP): A, mixture of N^{ϵ}-(6,8-disulfooctanoyl)-L-lysine (purple color with ninhydrin), L-aspartic acid and L-asparagine; B, N^{α}-(L-asparaginyl)-N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine (tan color with ninhydrin); C, (B) after treatment with LAP; D, N^{α}-(β-L-aspartyl)-N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine (blue color with ninhydrin), before and after treatment with LAP; E, N^{α}-(α-L-aspartyl)-N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine (purple color with ninhydrin); F, (E) after treatment with LAP. Approximately 0.015 μmole of each peptide was neutralized with sodium bicarbonate and then incubated with LAP in 0.02 *M* tris-(hydroxymethyl)-aminomethane buffer, *p*H 8.2, for 17 hr. at 37°. The incubation mixtures were subjected to paper electrophoresis in 1 *N* acetic acid, *p*H 2.3, for 4 hr. at 400 volts.

ref. 8). Confirmation of the structure assigned to peptide P-1 was obtained by synthesis of N^{α}-(β-L-aspartyl)-N^{ϵ}-(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. Peptide P-3A gave a yellow color with ninhydrin, which is indicative of an αβ-aspartyl derivative.[7,8] Further evidence supporting the structures assigned to peptides P-2C and P-3A was obtained by experiments with leucine aminopeptidase, which are discussed below. The nature of band P-3B is unknown. On complete hydrolysis it gave aspartic acid and lysine in a molar ratio of 0.3:1.0. There was insufficient material for further characterization.

Bands P-2(A+B) and P-5(A+B) (Table I) appeared to be mixtures of two components since glycine and aspartic acid were detected as N-terminal residues in each band. Band P-5(A+B) was separated into two components by treatment with 2,4-dinitrofluorobenzene followed by paper chromatography of the DNP derivatives with butanol–acetic acid–water (4:1:5) as the solvent system. The composition of the two DNP-peptides is shown in Table III. Although band P-2(A+B) was not separated into its components, evidence bearing on their structure was obtained by experiments with leucine aminopeptidase as described below.

Leucine aminopeptidase proved to be of value in establishing the structures of the aspartyl peptides. In model experiments it was shown that this enzyme did not attack N^{α}-(β-L-aspartyl)-N^{ϵ}-(6,8-disulfooctanoyl)-L-lysine but did attack N^{α}-(α-L-aspartyl)-N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine and N^{α}-(L-asparaginyl)-N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine, liberating aspartic acid and asparagine, respectively. The products were separated by paper electrophoresis at *p*H 2.3 (Fig. 3). The results of experiments with several of the isolated radioactive aspartyl peptides are summarized in Table V. Peptides P-1 and P-2C were not attacked by leucine aminopeptidase, indicating that these are β-aspartyl peptides. As mentioned above these two peptides gave a blue color with ninhydrin, an observation which is consistent with this conclusion. Peptide P-3A, which had the same composition as peptide P-2C (Table III) but migrated at a slower rate during electrophoresis (Fig. 1) and gave a yellow color with ninhydrin, was largely converted by incubation with leucine aminopeptidase into a substance which migrated at the same rate as peptide P-2C and gave a blue color with ninhydrin. It was suspected that this conversion was non-enzymatic and, indeed, when peptide P-3A was incubated with the alkaline buffer, peptide P-2C was produced. The synthetic α- and β-aspartyl peptides were not affected under the latter conditions. It is concluded from these results that peptide P-3A is an αβ-aspartyl peptide. Apparently, it is converted under alkaline conditions to the β-aspartyl peptide (P-2C) and a trace of the α-

(8) P. M. Bryant, R. H. Moore, P. J. Pimlott and G. T. Young, *J. Chem. Soc.*, 3868 (1959).

TABLE V

SUMMARY OF LEUCINE AMINOPEPTIDASE DEGRADATION OF S^{35}-LABELED 6,8-DISULFOÖCTANOYLPEPTIDES

Band no.	Treatment[a]	Radioactive peptide produced	Amino acid released	Linkage of aspartyl residue
P-1	LAP	No attack		β-
P-2 (A + B)	LAP	P-1, P-3C, X[b]	Asp,Gly(tr)[c]	α- in 2A $\alpha\beta$- in 2B
P-2C	LAP	No attack		β-
P-3A	LAP	P-2C, P-3C(tr)	Asp(tr),Ala(tr)	$\alpha\beta$-
P-3A	Buffer[d]	P-2C	None	
P-5 (A + B)	LAP	P-3C, P-2C(tr)	Asp,Ala,[e]Gly[e]	α- in 5A α- in 5B
K-1	LAP	P-1, X, P-3C(tr)	Thr,Asp(tr)	$\alpha\beta$-
K-1	$NaHCO_3$, then LAP[f]	P-1, P-3C(tr)	Thr,Asp(tr)	

[a] Approximately 0.015 μmole of each peptide was treated with leucine aminopeptidase (LAP) under the conditions described in legend of Fig. 3. The incubation mixtures were subjected to paper electrophoresis at *p*H 2.3 for 4 to 4.5 hr. [b] Peptide X appears to be α,β-Asp.ϵ-DSO-Lys (see text). [c] The letters "tr" indicate a small but significant amount of product. [d] 0.2 *M* tris buffer, *p*H 8.2. [e] Alanine and glycine were not completely separable under the conditions of electrophoresis used. [f] Peptide K-1 was heated with 1% aqueous sodium bicarbonate solution for 5 hr. at 100° prior to incubation with LAP.

aspartyl peptide, which in turn is attacked by leucine aminopeptidase. This interpretation is consistent with the observation of Swallow and Abraham[7] that N^{ϵ}-($\alpha\beta$-aspartyl)-L-lysine is readily converted by dilute alkali into a mixture of the α- and β-aspartyl peptides, with the latter isomer predominating.

Band P-5(A+B) when treated with leucine aminopeptidase gave aspartic acid, alanine, glycine, compound P-3C and a trace of peptide P-2C. The electrophoretic pattern of band P-5(A+B) was not affected by incubation with alkaline buffer alone. These results are best explained by assuming that peptides P-5A and P-5B, which were shown to be present in band P-5(A+B), are α-aspartyl peptides. A small amount of Gly.$\alpha\beta$-Asp.ϵ-DSO-Lys.-Ala may also be present in band P-5(A+B), which would account for the formation of a trace of peptide P-2C during incubation of band P-5(A+B) with leucine aminopeptidase, but not with buffer alone.

Band P-2(A+B) when incubated with leucine aminopeptidase gave aspartic acid, glycine, compound P-3C, peptide P-1 and a third radioactive spot (X) which migrated at a slower rate than peptide P-1 and gave a yellow color with ninhydrin. The electrophoretic pattern of band P-2(A+B) was not affected by incubation with alkaline buffer alone. When spot X was eluted and heated with 1% aqueous sodium bicarbonate solution for 5 hr. at 100°, it was converted to peptide P-1 and a trace of a substance which migrated at the same rate as synthetic N^{α}-(α-L-aspartyl)-N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine. The latter two peptides gave a blue and a violet color, respectively, with ninhydrin and were not affected by heating with 1% aqueous sodium bicarbonate solution. It appears from these data that peptide X is $\alpha\beta$-Asp.ϵ-DSO-Lys and that band P-2(A+B) is a mixture of α-Asp.ϵ-DSO-Lys and Gly.$\alpha\beta$-Asp.ϵ-DSO-Lys.

Incubation of peptide K-1 with leucine aminopeptidase gave threonine, peptide P-1, peptide X and traces of aspartic acid and compound P-3C. When peptide K-1 was heated with 1% aqueous sodium bicarbonate solution, followed by incubation with leucine aminopeptidase, peptide X was not present among the products, which consisted of threonine, peptide P-1 and traces of aspartic acid and compound P-3C. These results indicate that peptide K-1 is Thr.$\alpha\beta$-Asp.ϵ-DSO-Lys and is converted to Thr.β-Asp.ϵ-DSO-Lys and a trace of Thr.α-Asp.ϵ-DSO-Lys by heating under alkaline conditions. Attack of the latter peptide by leucine aminopeptidase would account for the presence of trace amounts of aspartic acid and compound P-3C in the incubation mixtures.

Additional evidence concerning the structure of peptide K-2 was obtained by treatment with carboxypeptidase. Equimolar amounts of leucine and valine were released. The radioactive peptide remaining gave α-DNP-lysine and valine in equimolar amounts when analyzed by the 2,4-dinitrophenyl technique. The data indicate that peptide K-2 is ϵ-DSO-Lys.Val.(Val,Leu). There was insufficient material for further characterization.

A partial acid hydrolysate of peptide K-3 was treated with 2,4-dinitrofluorobenzene, and the ether-soluble and water-soluble DNP derivatives were separated by paper chromatography and paper electrophoresis, respectively. The ether-soluble DNP derivatives consisted of DNP-threonine, DNP-aspartic acid, DNP-valine, DNP-leucine, DNP-glutamic acid and two DNP peptides which were characterized as DNP-Thr.Asp and DNP-Val.(Val,Leu). The major water-soluble DNP derivative, which was radioactive, was characterized as DNP-Thr.Asp.ϵ-DSO-Lys. On the basis of these data, that given in Table IV, and the structures assigned to peptides K-1 and K-2, it appears that the structure of peptide K-3 is Thr.Asp.ϵ-DSO-Lys.Val.(Val,Leu).Glu.

The data presented in this paper indicate that the pyruvate dehydrogenation complex contains the sequence Gly.Asp.ϵ-Lipoyl-Lys.Ala and that the α-ketoglutarate dehydrogenation complex contains the sequence Thr.Asp.ϵ-Lipoyl-Lys.Val.(Val,Leu).-Glu. Whereas it seems most likely that the aspartyl residue in these two sequences is the α form, the possibility cannot be excluded that it may be partly in the β or $\alpha\beta$ form. The possibility also has to be considered that an asparaginyl rather than as aspartyl residue is present. However, it should be emphasized in this connection that asparagine was not detected among the amino acids

released by the action of leucine aminopeptidase on the isolated 6,8-disulfoöctanoylpeptides (Table V).

Experimental

Materials and Methods.—The highly purified pyruvate and α-ketoglutarate dehydrogenation complexes used in this work were prepared as described previously[8,9] from extracts of *E. coli* (Crookes strain) cells which had been grown on a medium containing DL-lipoic acid $-S_2^{35}$ (4.46 × 10^4 c.p.m. per μg.). The enzyme preparations, corresponding to Precipitates B and A of the purification procedure, contained, respectively, 1.46 and 0.98 μg. of bound radioactive lipoic acid per mg. of protein. Crystalline carboxypeptidase was purchased from Worthington Biochemical Corporation. Leucine aminopeptidase was a generous gift from Dr. Austen Riggs, and was purified according to the procedure of Hill, *et al.*,[10] omitting the final starch zone-electrophoresis step.

Methods pertaining to paper chromatography and paper electrophoresis have been described.[3] Ninhydrin-reactive substances were located by spraying the air-dried papers with a solution of 0.2% ninhydrin in water-saturated butanol, followed by rapid drying at 100–120°.[8]

The synthesis of N^{ϵ}-(6,8-disulfoöctanoyl)-L-lysine was reported previously.[3] The synthesis of N^{α}-(α-L-aspartyl)-N^{ϵ}-lipoyl-L-lysine, N^{α}-(β-L-asparty1)-N^{ϵ}-lipoyl-L-lysine and N^{α}-(L-asparaginyl)-N^{ϵ}-dihydrolipoyl-L-lysine is described in the accompanying paper.[11] These three compounds were oxidized to the corresponding 6,8-disulfoöctanoyl derivatives with performic acid.[3] The products, which were hygroscopic solids, gave single ninhydrin-positive spots (*cf.* Fig. 3).

Isolation of Radioactive 6,8-Disulfoöctanoylpeptides. (a) From Partial Hydrolysates of Oxidized Pyruvate Dehydrogenation Complex.—A 580-mg. sample of the pyruvate dehydrogenation complex was treated with 19 ml. of performic acid reagent[8,12] at 4° for 16 hr. The clear solution was lyophilized, the residue was dissolved in 41 ml. of 12 *N* hydrochloric acid and the solution was heated under a nitrogen atmosphere for 6 days at 37°. The hydrolysate was evaporated *in vacuo* and the residue was dried over solid potassium hydroxide. This material was dissolved in 20 ml. of water, and the solution was extracted with 20-ml. portions of ethyl acetate and chloroform to remove non-radioactive, yellow, fluorescent material. Recovery of radioactivity at this stage was essentially quantitative. The aqueous layer was lyophilized and the residue was dissolved in 4 ml. of water and applied to a column (29 ml.) of Dowex 50W-X8 (200 to 400 mesh) in the hydrogen cycle. Water was passed through the column and 2-ml. fractions were collected. Fractions 7–10, which contained 96% of the radioactivity, were combined and lyophilized. The residue was dissolved in a minimal volume of water and the solution was applied as a 30-cm. band 10 cm. from the ends of two 35 × 57 cm. sheets of Whatman No. 3MM paper. Chromatography was carried out by the descending technique for 91 hr. with butanol–acetic acid–water (2:1:1) as the solvent system. The solvent was allowed to drip from the bottom of the paper into a trough during development. Approximately 4% of the radioactivity was present in 179 ml. of effluent, which was discarded. A radioautograph of the chromatogram showed four major bands at distances of 25 cm. (P-1), 30 cm. (P-2), 32 cm. (P-3) and 41 cm. (P-4) from the origin. The bands were cut out and eluted with 2 ml. of water. Recovery of radioactivity in the eluates was 9, 12, 30 and 17%, respectively. Each eluate was concentrated and applied as a 17-cm. band 5 cm. from the cathode ends of 22.4 × 30.6 cm. sheets of Whatman No. 3MM paper. Electrophoresis was carried out in a Spinco model R apparatus at room temperature. The electrolyte used was 1 *N* acetic acid, *p*H 2.3. A potential difference of 400 volts was applied for 4 hr. The radioactive bands, located by radioautography, were cut out and eluted with water. Each eluate was concentrated and passed through a column (1.5 ml.) of Dowex 50 in the hydrogen cycle. Aliquots of the radioactive effluents were subjected to paper electrophoresis. The electrophoretic pattern is shown in Fig. 1. Each radioactive spot coincided with a single ninhydrin-positive spot, with the exception of band P-3B, which showed a non-radioactive, ninhydrin-positive spot. The latter substance appeared to be cysteic acid, as indicated by two-dimensional paper chromatography.[3] It was separated from the radioactive component in band P-3B by repeating the electrophoresis step. Over-all recoveries of the radioactive 6,8-disulfoöctanoylpeptides are given in Table I (Expt. 1).

In a second experiment a 440-mg. sample of the pyruvate dehydrogenation complex was oxidized with performic acid, and the oxidized protein was partially hydrolyzed with 32 ml. of 12 *N* hydrochloric acid–acetic acid (1:1) for 5 days at 37°. The hydrolysate was worked up as described above. Recoveries of readioactive peptides are given in Table I (Expt. 2). The electrophoretic mobility of band P-5(A+B) relative to the mobilities of the peptides obtained in Expt. 1 is illustrated in Fig. 1.

(b) From Partial Hydrolysates of Oxidized α-Ketoglutarate Dehydrogenation Complex.—A preparation of the α-ketoglutarate dehydrogenation complex (492 mg. of protein) was oxidized with performic acid and then hydrolyzed with 35 ml. of 12 *N* hydrochloric acid for 4 days at 37°. The partial hydrolysate was treated with Dowex 50 and then subjected to large scale paper chromatography as described above. A radioautograph of the chromatogram showed a single radioactive band at a distance of 32 cm. from the origin. This band was eluted with water (recovery of radioactivity, 38%) and subjected to paper electrophoresis at *p*H 2.3. A single radioactive, ninhydrin-positive band (K-1) was detected. The effluent (118 ml.) from the paper chromatogram contained 48% of the radioactivity. It was evaporated to dryness and the residue was subjected to paper electrophoresis at *p*H 2.3. Two major radioactive bands (K-2 and K-3) were detected on the electrophoretogram. The bands were eluted with water and aliquots were subjected to paper electrophoresis. The only ninhydrin-positive spots present coincided with the radioactive spots on the radioautograph. The electrophoretic pattern is shown in Fig. 2. Over-all recoveries of the radioactive peptides are shown in Table II (Expt. 1).

A second sample of the α-ketoglutarate dehydrogenation complex (250 mg. of protein) was hydrolyzed with 12 *N* hydrochloric acid–acetic acid (1:1) for 4 days at 37°. The hydrolysate was worked up as described above, with the exception that development of the paper chromatogram was carried out for only 24 hr. A radioautograph prepared from the chromatogram showed two major radioactive bands at distances of 9 cm. and 29 cm. from the origin. These bands were cut out, eluted with water and purified further by paper electrophoresis at *p*H 2.3. The mobilities of these bands and their amino acid composition corresponded to those of peptides K-1 and K-3. Recoveries of radioactivity are given in Table II (Expt. 2).

Amino Acid Analyses.—Approximately 0.05 μmole of each radioactive peptide was heated with 0.2 ml. of redistilled, azeotropic 5.7 *N* hydrochloric acid for 24 hr. at 105° under nitrogen in a sealed tube. The hydrolysate was evaporated under a stream of nitrogen and then dried *in vacuo* over solid potassium hydroxide. For qualitative amino acid analysis the residue was subjected to two-dimensional paper chromatography with butanol–acetic acid–water (4:1:5) in the first dimension and water-saturated phenol–5% ammonia in the second dimension. Quantitative amino acid analyses and amino end group analyses were performed by the method of Sanger,[6] essentially as given by Fraenkel-Conrat, *et al.*[13] The DNP amino acids were separated by chromatography in the *t*-amyl alcohol–phthalate buffer system, quantitatively eluted from the paper and estimated spectrophotometrically. Toward the end of this investigation it was possible, through the kindness of Dr. Austen Riggs, to determine the amino acid composition of several of the peptides by means of a Spinco amino acid analyzer. The results are shown in Tables III and IV.

(9) M. Kolke, L. J. Reed and W. R. Carroll, *J. Biol. Chem.*, **235**, 1924 (1960).

(10) R. L. Hill, D. H. Spackman, D. M. Brown and E. L. Smith, *Biochem. Preparations*, **6**, 35 (1958).

(11) K. Daigo, W. T. Brady and L. J. Reed, *J. Am. Chem. Soc.*, **84**, 662 (1962).

(12) E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

(13) H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, in D. Glick (Editor), "Methods of Biochemical Analysis," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1955, p. 359.

To confirm the identity of α-DNP-lysine, paper electrophoresis in 1 *N* acetic acid, *p*H 2.3, was used. In the Spinco model R apparatus, with a potential difference of 400 volts, α-DNP-lysine migrated a distance of 7.9 cm. toward the cathode in 3 hr., whereas ε-DNP-lysine migrated a distance of 6.1 cm. To confirm the identity of DNP-leucine, the DNP amino acid was converted to the free amino acid with concentrated ammonium hydroxide at 105°,[14] and the latter was identified as leucine by paper chromatography in the *t*-amyl alcohol–phthalate buffer system.

Characterization of Band P-5(A+B).—A 0.063-μmole sample of this material was treated with 2,4-dinitrofluorobenzene, and the reaction mixture was extracted with ether and passed through a small column of Dowex 50. The effluent was concentrated and subjected to descending paper chromatography for 48 hr. with butanol–acetic acid–water (4:1:5). Two yellow, radioactive bands were detected at distances of 15.5 cm. (5A) and 17.5 cm. (5B) from the origin. Recovery of radioactivity in the eluates of these bands was 0.014 μmole (22%) and 0.026 μmole (42%), respectively. Each DNP-peptide was hydrolyzed with 5.7 *N* hydrochloric acid at 105° and the DNP amino acid (ether-soluble) was identified by paper chromatography and analyzed quantitatively. The results are shown in Table III.

Characterization of Peptide K-2.—A mixture of 0.038 μmole of peptide K-2 and 6×10^{-4} μmole of carboxypeptidase was adjusted to *p*H 8.0 and then incubated at 25° for 1 hr. The incubation mixture was treated with 2,4-dinitrofluorobenzene.[5] Analysis of the ether-soluble DNP amino acids showed 0.032 μmole of DNP-leucine and 0.034 μmole of DNP-valine. The aqueous layer was concentrated and subjected to paper electrophoresis at *p*H 2.3 for 3 hr. A major yellow, radioactive band was detected at a distance of 16.3 cm. toward the anode. This band was cut out, eluted with water, and the eluate was passed through a small column of Dowex 50. Recovery of radioactivity was 56% (0.021 μmole). The eluate was evaporated to dryness and the residue was heated with 5.7 *N* hydrochloric acid for 15 hr. at 105°. The hydrolysate was evaporated to dryness and the residue was subjected to paper chromatography in the *t*-amyl alcohol-phthalate buffer system together with authentic samples of lysine, valine, α-DNP-lysine and ε-DNP-lysine. α-DNP-lysine was detected on the chromatogram. Quantitative analysis of this spot showed 0.020 μmole. The chromatogram was then sprayed with a solution of 0.2% ninhydrin in water-saturated butanol, and the color was allowed to develop at room temperature in the dark for 3 days. A single ninhydrin-positive spot was detected, corresponding in R_f to valine. This spot was cut out, eluted with 50% ethanol in water, and its concentration estimated as 0.023 μmole on the basis of the absorbancy at 570 mμ.[7]

Characterization of Peptide K-3.—A 0.2-μmole sample of peptide K-3 was dissolved in 0.2 ml. of 5.7 *N* hydrochloric acid and the solution was allowed to stand in a sealed tube for 5 days at 37°. The hydrolysate was evaporated to dryness and the residue was treated with 2,4-dinitrofluorobenzene. The ether-soluble DNP derivatives were subjected to two-dimensional paper chromatography[13] with the toluene–chloroethanol–pyridine–aqueous ammonia system in the first dimension and 1.5 *M* phosphate buffer, *p*H 6, in the second dimension. A major yellow spot and six yellow spots of lesser intensity were detected. Each spot was cut out, eluted and then hydrolyzed with 5.7 *N* hydrochloric acid at 105°. The ether-soluble DNP amino acids and the water-soluble amino acids were identified by paper chromatography in the *t*-amyl alcohol–phthalate buffer system and the butanol–acetic acid–water (4:1:5) system, respectively. Five of the yellow spots of lesser intensity appeared to be DNP-glutamic acid, DNP-aspartic acid, DNP-threonine, DNP-valine and DNP-leucine. The sixth spot appeared to be a dipeptide possessing the structure DNP-Thr.Asp. The major yellow spot appeared to be a tripeptide possessing the structure DNP.Val.(Val,-Leu). Quantitative analysis of this peptide showed the following molar ratios: DNP-Val, 1.0; Val, 1.0; Leu, 1.2.

The aqueous layer from the 2,4-dinitrofluorobenzene treatment was concentrated and subjected to paper chromatography for 68 hr. in the butanol–acetic acid–water (4:1:5) system. A major and two minor radioactive bands were detected at distances of 14.5, 11.5 and 16.5 cm., respectively, from the origin. The major band was eluted and the eluate was passed through a column of Dowex 50. Recovery of radioactivity in the effluent was 33%. A single, yellow, radioactive component was detected by paper electrophoresis at *p*H 2.3. Quantitative analysis of this peptide showed the following molar ratios: DNP-Thr, 0.97; Asp, 1.1; Lys, 1.0. This peptide therefore appears to possess the structure DNP-Thr.Asp.ε-DSO-Lys.

Acknowledgments.—We are indebted to Dr. Masahiko Koike and Mrs. Elizabeth Thompson for the preparations of α-keto dehydrogenation complexes containing bound lipoic acid $-S_2^{35}$.

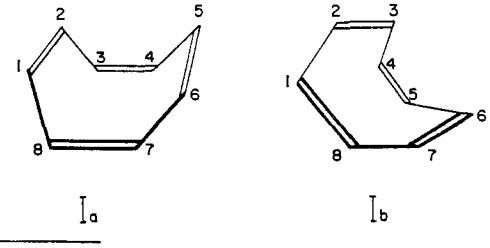
(14) A. G. Lowther, *Nature*, **167**, 767 (1951).

COMMUNICATIONS TO THE EDITOR

THE RATE OF BOND CHANGE IN CYCLOÖCTATETRAENE

Sir:

Cycloöctatetraene(I) is known[1] to have alternate single and double bonds in a tub-shaped (D_{2d}) non-aromatic structure (Ia). Structure Ib is equivalent to Ia in the absence of labelling[2]; pairs of carbon atoms which are doubly bonded in Ia are singly bonded in Ib and *vice versa*. The bond change from Ia to Ib almost certainly involves a strained planar intermediate or transition state with limited resonance stabilization, as a result of the unfavorable[3] number (8) of π electrons in I. Thus, an appreciable energy barrier would be expected to separate Ia from Ib.



Ia Ib

(1) I. L. Karle, *J. Chem. Phys.*, **20**, 65 (1952); W. B. Person, G. C. Pimentel and K. S. Pitzer, *J. Am. Chem. Soc.*, **74**, 3437 (1952).

(2) In a monosubstituted cycloöctatetraene, 1a and 1b are enantiomers. Attempts to resolve such compounds have been unsuccessful (A. C. Cope, M. Burg and S. W. Fenton, *ibid.*, **74**, 173 (1952), A. C. Cope and M. R. Kinter, *ibid.*, **73**, 3424 (1951). The present work shows that the rate of racemization would be much too high to allow resolution. It is proposed to examine the n.m.r. spectra of such compounds at low temperature.

(3) See D. P. Craig in "Non-benzenoid Aromatic Compounds," edited by David Ginsburg, Interscience Publishers, Inc., New York, N. Y., 1959, p. 1.