tritylated to peptide esters, or first saponified and then detritylated to give the free peptides. Examples of these procedures are given below; the peptides and intermediates prepared are listed in Table V.

A solution containing 3.2 g. (0.01 mole) of tritylglycine, 30 ml. of dry chloroform and 2 g. (0.02 mole) of dry triethylamine was cooled to 0°, and 1.08 g. (0.01 mole) of ethyl chloroformate was added. After 15 minutes 2.0 g. (0.01 mole) of glycine benzyl ester hydrochloride dissolved in 15 ml. of chloroform with 1 g. (0.01 mole) of triethylamine was added. Coupling proceeded with the evolution of CO_2. The solution was kept at room temperature for 30 minutes and then washed successively with dilute acetic acid, twice with dilute aqueous diethylamine solution and with water. It was dried with sodium sulfate and evaporated to dryness *in vacuo*. To the residue ethanol was added and the mixture distilled *in vacuo*, yielding tritylglycylglycine benzyl ester.[23] This ester was (a) detritylated with alcoholic hydrogen chloride, as described previously, to give glycylglycine benzyl ester hydrochloride; or (b) saponified with alcoholic potassium hydroxide. After 30 minutes, 80 ml. of water was added and the solution acidified with acetic acid. The precipitated tritylglycylglycine was recrystallized from alcohol. It was detritylated with acetic acid or by catalytic hydrogenation to yield glycylglycine.

N,N-Bistritylglycyl-L-cystine dimethyl ester[23,24] (sirup), dissolved in 50 ml. of methanol and 4 ml. of hydrazine hydrate, was stored in the ice-box for 48 hours. The precipitated dihydrazide was recrystallized from methanol.

N-Trityldiethylamine.—To a solution containing 2.8 g. (0.01 mole) of trityl chloride in 20 ml. of chloroform, was added 4 ml. of diethylamine. After one hour the solution was washed twice with water and dried with sodium sulfate. The crystalline residue, which was obtained after distillation of the solvent *in vacuo*, was recrystallized from petroleum ether; yield 2.2 g. (70%) needles, m.p. 107°.

Anal. Calcd. for $C_{23}H_{25}N$: C, 87.6; H, 8.0; N, 4.4. Found: C, 87.4; H, 8.3; N, 4.3.

(23) It was obtained in the same yield when tritylglycyl chloride-hydrochloride (0.01 mole) was added to a chloroform solution containing at least 0.03 mole of amino acid ester.

(24) This ester was prepared by coupling tritylglycine with 1.7 g. (0.005 mole) of L-cystine dimethyl ester hydrochloride dissolved in 15 ml. of chloroform with 1 g. of triethylamine as described above.

ATHENS, GREECE

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF MARYLAND]

The Purification of Hog Kidney D-Amino Acid Oxidase

BY F. P. VEITCH AND ROBERT MCCOMB[1]

RECEIVED JUNE 29, 1955

This paper describes the purification of D-amino acid oxidase by application of the principle of heat stabilization of enzyme by a competitive inhibitor. D-Amino acid oxidase is exceptionally stable to temperatures of 57° for 15 minutes in the presence of the ammonium salt of *m*-toluic acid (0.0178 *M*) at *p*H 5.2. In the absence of inhibitor 81% of the enzyme is destroyed under these same conditions. By the use of this technique a one hundred-fold enrichment, with a 67% yield, is accomplished in two steps. The product is pure as judged by several tests of homogeneity.

The stabilizing influence of substrates, coenzymes and inhibitors upon enzymes is well known. Advantage has been taken of this effect in the differential heat denaturation of contaminating proteins during the course of enzyme purification.[2]

In the research reported here, *m*-toluic acid, a competitive inhibitor of D-amino acid oxidase[3] has been found to stabilize the enzyme to the effect of temperature to a very marked degree (Table I).

TABLE I

EFFECT OF TIME OF HEATING, TEMPERATURE AND *p*H OF HEATING ON THE STABILITY OF D-AMINO ACID OXIDASE IN THE PRESENCE OF 0.0178 *M* AMMONIUM TOLUATE

Temp., °C.	Time, min.	*p*H	Specific activity, μl. O_2/10 min./mg. protein	Yield, %
45–50	5	5.1	134	..
55–60	5	5.1	188	..
67–70	5	5.1	177	..
57	10	5.1	930	79
57	15	5.1	1000	77
57	20	5.1	850	41
57	15	4.6	..	4
57	15	5.0	..	42
57	15	5.1	1250	50
57	15	5.15	1500	70
57	15	5.2	1390	67
57	15	5.6	..	63
57	15	7.5	..	6

(1) Monsanto Fellow, 1953–1954.

(2) (a) K. Burton, *Biochem. J.*, **48**, 458 (1951); (b) T. P. Singer and E. B. Kearney, *Arch. Biochem.*, **29**, 190 (1950).

(3) G. R. Bartlett, THIS JOURNAL, **70**, 1010 (1948).

We ascribe this stabilizing effect to the binding of the functional groups of the enzyme by the inhibitor in a manner similar to, but more pronounced than, the binding of normal substrates. This belief is strengthened by a comparison of the Michaelis constants of the enzyme–inhibitor and enzyme–substrate complexes (Table II). Further, we have found that weaker competitive inhibitors are less effective in protecting the enzyme to heat denaturation (Table II).

TABLE II

PROTECTION TO HEAT DENATURATION OFFERED BY VARIOUS COMPETITIVE INHIBITORS

Inhibitor	K_i/K_m[a]	% of original activity after heating and removal of inhibitor
m-Aminobenzoic acid	29×10^{-2}	66
Benzoic acid	3.47×10^{-2}	68
m-Toluic acid	1.15×10^{-2}	73
None	.....	19

[a] Bartlett[3] reported the effectiveness of various substituted benzoic acids as inhibitors of D-amino acid oxidase as molar concentrations giving 50% inhibition. Using the value of $K_m = 6.1 \times 10^{-3}$ for alanine obtained by Hellerman,[4] and substituting the inhibitor and substrate concentrations used by Bartlett in the equation $V/V_i = 1 + K_m/K_i\ (I/K_m + S)$ we have calculated the K_i values used above. Terms are defined as: V = velocity of uninhibited reaction (μl. O_2/10 min./mg. protein); V_i = velocity of inhibited reaction (μl. O_2/10 min./mg. protein); K_m = Michaelis constant; K_i = inhibitor constant; S = concentration of substrate in moles; I = concentration of inhibitor in moles.

(4) L. Hellerman, A. Lindsay and M. R. Bovarnick, *J. Biol. Chem.*, **163**, 553 (1946).

We have investigated the homogeneity of our purest enzyme[5] preparation by several procedures: *i.e.*, the specific property test,[6] column chromatography, filter paper electrophoresis and conventional electrophoresis. Some discussion of the results of these homogeneity tests is in order.

Specific Property Test.[5]—The results of the specific property test are shown in Fig. 1. In this test of homogeneity the enzyme was subjected to successive fractional precipitation with ammonium sulfate over the range of 0.2 to 0.4 saturation. The activities and protein content of the several fractions was determined and these values used to prepare a graph of activity *versus* protein content. If the enzyme is homogeneous the activities of all fractions should, within experimental error, fall upon a line determined by the origin and a point described by the activity and protein content of the purified enzyme. The presence of less soluble impurities will shift this line to the right. As can be seen from the plot (circles with horizontal cross hatching) a small amount of less soluble impurity remains even after discarding the fraction insoluble in 0.2 saturated ammonium sulfate. We feel that this is due to denaturation of the pure protein at low salt concentrations during the course of the experiment. We have observed repeatedly that this enzyme is quite unstable in solutions of low ionic strength.

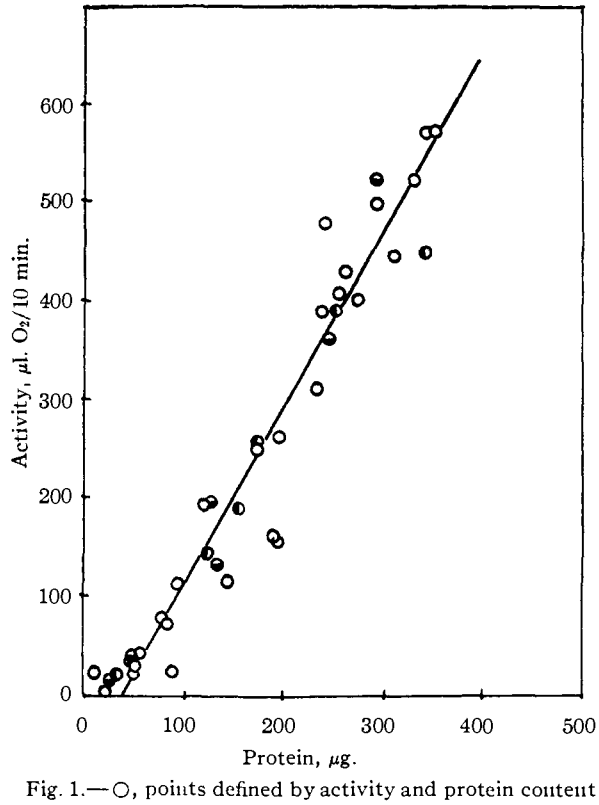

Fig. 1.—○, points defined by activity and protein content and precipitates obtained between 0.2 and 0.4 saturation with respect to ammonium sulfate. Enzyme solution used had been heated in the presence of ammonium toluate and precipitated once at *p*H 8.0 with 0.4 sat. ammonium sulfate; ◑, same as above except enzyme purified as described in Experimental: Purification of D-amino acid oxidase. ⊖, same as ◑ except the enzyme had been further purified by discarding all material insoluble at 0.2 sat. with respect to ammonium sulfate. Line drawn by the method of least squares.

Column Chromatography.—The results of the chromatographic treatment of the purified enzyme are shown in Fig. 2. The fact that the active enzyme was eluted as a single component, and that this activity paralleled the appearance of protein in the eluate, indicates an homogeneous preparation.

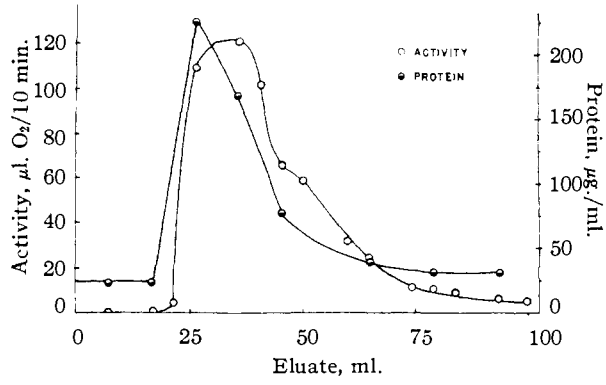

Fig. 2.—Chromatography of D-amino acid oxidase on Hyflo Super-Cel, elutant *M*/50 pyrophosphate.

Filter Paper Electrophoresis.—The results of filter paper electrophoresis are shown in Fig. 3, which reveals the presence of a single protein band. In addition, when similar electrophoretic strips were cut into segments and the activity resident in these strips determined, the position of maximum activity and protein content coincided. This indicates an homogeneous preparation.

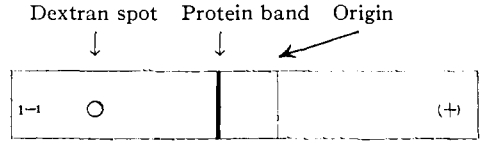

Fig. 3.—Paper electrophoresis of D-amino acid oxidase; scale drawing of actual strip: buffer, *M*/10 alanine *p*H 8.5; time, 12 hr.; voltage, 200 v.; current, 1.5 ma.; 0.8% purified enzyme in *M*/10 alanine, *p*H 8.5; paper, Whatman #2.

Conventional Electrophoresis.—Electrophoretic patterns were obtained at three different *p*H values, *p*H 4.86, 7.5 and 8.4 (Fig. 4 and Table III). Unfortunately the solubility of this protein is low, particularly in acid solutions. This made the attainment of good electrophoretic patterns difficult. The best results were obtained at *p*H 7.5 where the protein was soluble to the extent of 0.8%. In experiments of three-hour duration at both *p*H 4.86 and 8.4, the enzyme was found to migrate as a single component. However, at *p*H 7.5, when the dura-

(5) The term enzyme will be used in this paper to denote the holoenzyme. Since no attempt is made in this purification to separate the apoenzyme from coenzyme this terminology will not be rigorously correct in all cases because some loss of coenzyme is unavoidable. Unless otherwise stated, activity determinations are all performed with sufficient coenzyme (Flavine adeninedinucleotide) present to saturate fully the apoenzyme.

(6) J. S. Falcoiier and D. B. Taylor, *Biochem. J.*, **40**, 831 (1946).

TABLE III

MOBILITIES AND SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED DURING ELECTROPHORESIS

Enzyme soln.	Time, sec.	Mobility, cm./sec. × 10^5 Ascend. Lead	Ascend. Lag	Descend. Lead	Descend. Lag	Position of sample (see Fig. 4)	Specific activity, μl. O_2/10 min./mg. protein Without FAD	With FAD
0.8% in 0.084 molar phosphate, *p*H 7.5	7,800	..	3.03	3.80	..	A_1	240	749
	13,500	3.87	3.13	3.93	2.73	A_2	860	1840
	22,860	3.87	3.18	3.85	2.70	A_3	1330	1400
	29,040	3.66	2.95	3.54	2.52	D_1	..	0
		3.80 av.	3.07 av.	3.78 av.	2.65 av.	D_2	140	785
						D_3	1210	1490
						Control	..	1330

tion of the experiment was prolonged to six hours, a second component appeared. Samples of the components in each of the three experiments were removed from the cell (see Experimental) and the activity of these samples compared with that of appropriate controls. In the case of the two three-hour experiments, fractions removed from the cell were of the same specific activity as the control. In the case of the six-hour experiment, however, the leading peak in the ascending arm and the trailing peak in the descending arm were less active than either the main peak or the control. The specific activity of the material in the main peak of the ascending arm, in the six-hour experiment compared favorably with the specific activity of the best enzyme preparation produced in this Laboratory. The calculated mobilities of the leading peaks in the two arms were in good agreement (Table III). The agreement between the mobilities of lagging peaks was not too good but the error was in the right direction. It has been found generally in electrophoretic work that the mobility of components in the descending arm of the cell is five to ten per cent. lower than in the ascending arm.[7]

The presence of enzymatic activity in the minor peaks coupled with the fact that the smaller peak leads in the ascending and lags in the descending arm reveals the anomalous behavior of this protein. A possible explanation of this behavior is that the enzyme adjacent to the buffer boundary dissociates into smaller fragments of differing mobilities. Experimental evidence supports one such dissociation, that of coenzyme splitting away from apoenzyme. The results in Table III, where activities are recorded for various samples with and without added FAD,[8] show that the degree of saturation with respect to FAD was always high in the main peak and fell off as the buffer boundary was approached. The low specific activity of the minor peak samples can be accounted for by denaturation of the enzyme in the dilute, FAD free, areas prior to the activity determination.

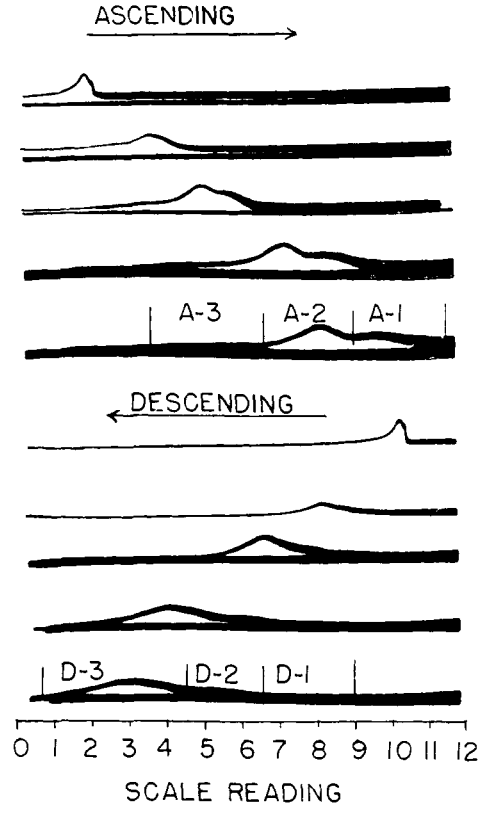

Fig. 4.—Electrophoresis of D-amino acid oxidase; see Table III for conditions.

Experimental

Reagents and Apparatus.—The source of the enzyme used in this investigation was a defatted and 37° desiccated hog kidney powder marketed by the Viobin Corporation of Monticello, Ill. All reagents were Eastman Kodak "ACS" grade or equivalent unless otherwise specified. "Hyflo Super-Cel" was obtained from the Johns-Manville Corporation. Permutit-A was obtained from the Permutit Company, Birmingham, New Jersey. Crystalline egg albumin used in the standardization of the protein determinations was prepared in this Laboratory. Protein determinations were made by the method of Lowry,[9] which was checked by Kjeldahl analyses of the standard egg albumin. Activity determinations were carried out with the aid of the conventional Warburg apparatus. Electrophoretic studies were made with the Aminco–Stern electrophoresis apparatus.[10]

Preparation of Crude Enzyme Extracts.—Crude extracts of the enzyme were prepared as described in the first step of Purification of D-amino acid oxidase, described below. The crude enzyme was stable for 24 hours if kept at 0°. The preparation lost some 13% of its original activity if

(7) D. M. Greenburg, "Amino Acids and Proteins," Charles C Thomas, Publisher, Springfield, Ill., 1951, pp. 506.

(8) Flavine adeninedinucleotide.

(9) O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(10) We are indebted to Dr. Singh Chen Chang Live Stock and Sanitary Laboratories, University of Maryland, for the use of this instrument and for technical assistance.

kept at room temperature for this time. This preparation had a specific activity of 6.4 μl. O_2/10 min./mg. protein. This figure must be multiplied by two to obtain the actual volume of oxygen taken up since catalase is present in these crude extracts.

Standard Activity Determination.—The activity of all fractions and of the purified enzyme was determined by measuring the oxygen uptake per mg. of protein for a ten-minute period. Activity measurements were made under conditions where activity was proportional to enzyme concentration. The coenzyme concentration of added crude FAD solutions was determined by the method of Warburg and Christian,[11] sufficient excess of coenzyme being added in the standard activity determinations to saturate fully the apoenzyme.

The procedure used in these activity measurements is described below. In the main body of a Warburg flask was placed 1.0 ml. of enzyme solution containing such a concentration of enzyme as to give an oxygen uptake of between 40 and 80 μl. of gas in 10 min. One ml. of crude coenzyme solution, containing sufficient coenzyme to saturate fully the enzyme used, was also added to the main body of the flask. The side arm was charged with 1.0 ml. of a freshly prepared $M/20$ pyrophosphate buffer (pH 8.3) which was in addition 0.138 M with respect to DL-alanine. After equilibration the reaction was started by tipping in the substrate, and the oxygen uptake for the first ten minutes recorded. It was not found necessary to use either sulfuric acid or sodium hydroxide in the center well because of the high solubility in the reaction medium of the two gases produced (ammonia and carbon dioxide).

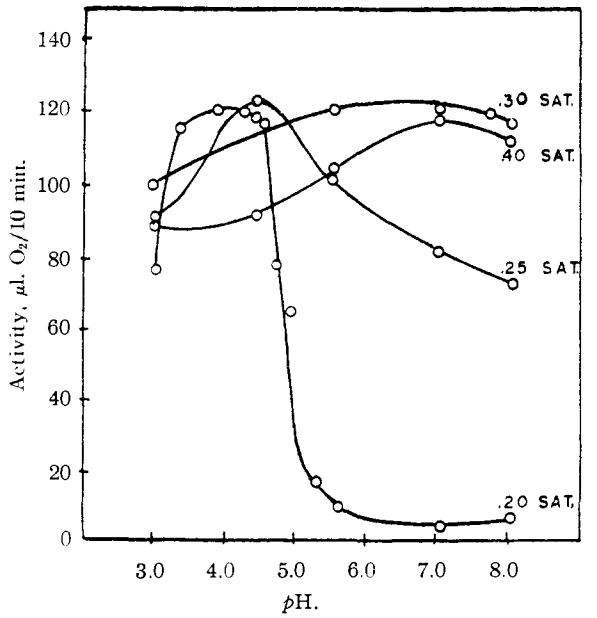

Fig. 5.—Optimum pH for precipitation of D-amino acid oxidase with respect to yield.

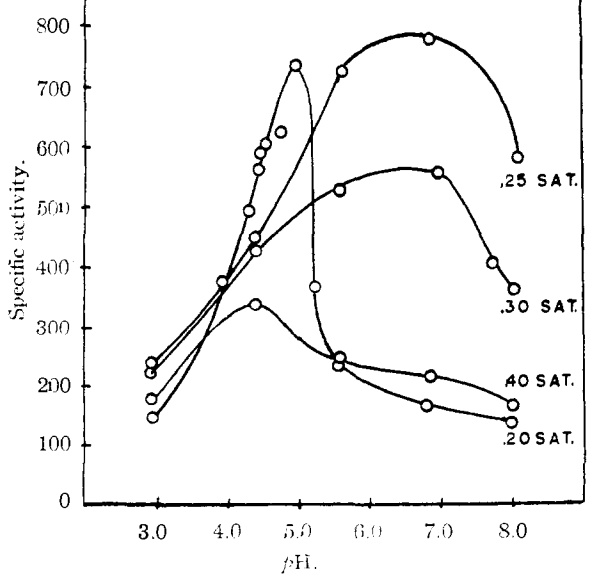

Fig. 6.—Optimum pH for precipitation of D-amino acid oxidase with respect to specific activity.

(11) O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938).

Preparation of Crude Coenzyme Solution.—Hog kidney powder was extracted for half an hour with 10 times its weight of boiling methanol. The mixture was filtered and the solid re-extracted under the same conditions. The methanol-insoluble residue was dried in an oven at 100° for one hour and then broken up to give a dry tan powder. This powder was extracted with 20 times its weight of $M/10$ pyrophosphate buffer (pH 8.3) for half an hour, and the clear yellow extract collected by filtration. The coenzyme solution, which had no enzymatic activity, was stored at 0° and always used within 12 hours after preparation.

Optimum Temperature, Time and pH of Differential Heat Denaturation.—In these experiments 20–50 ml. portions of crude hog kidney extract were made 0.018 M with respect to ammonium toluate and subsequently heated at various temperatures, times and pH values. After the heating period the solutions were passed through a Permutit-A column to remove toluate ion, and in some cases subjected to two ammonium sulfate precipitations as described below in the purification scheme.

Purification of D-Amino Acid Oxidase.—To 500 g. of hog kidney powder was added 6 l. of distilled water, and the mixture allowed to stand at room temperature for one-half hour with occasional stirring. The mixture was then filtered through a large bench-type Büchner funnel, the whole operation taking about two hours. The filter cake was re-extracted with 4 l. of distilled water and filtered. The two extracts were combined to give 8300 ml. of a dark brown solution. One ml. of this solution contained 14.40 mg. of protein and catalyzed an oxygen uptake of 92 μl. of oxygen in ten minutes in a standard activity determination. To 8300 ml. of this crude extract was added 2800 ml. of 0.072 M ammonium toluate solution previously prepared from m-toluic acid. The pH of the extract was then adjusted to 5.2 with 65 ml. of N hydrochloric acid. The solution, which now contained large amounts of white flocculent precipitate, was heated in one-liter batches for 15 min. in a 57° constant temperature bath. The solution was stirred vigorously for the first 6 minutes. In this period the temperature within the flask rose to bath temperature, and the heating was continued an additional 9 min. without stirring. The batches were cooled for one-minute in an ice-bath, filtered with the aid of suction, and the clear yellow filtrate transferred immediately to a 32 × 800 mm. column charged with Permutit-A to remove toluate ion. This moderately basic anion exchanger had been previously converted to the bicarbonate form by passing 2 l. of saturated sodium bicarbonate through the column charged with the hydroxide form of the resin. The column was further washed with several liters of distilled water before use. The rate of passage of the extract through the column was 1 l./hr. The whole operation was carried out in a cold room at 0°. The pH of the material which had passed through the column was usually about 7.5.

At this point in the purification it was found that a small amount of ammonium m-toluate was still present, particularly if passage through the column was too rapid. The residual m-toluate could be removed conveniently by two ammonium sulfate precipitations (precipitates I and II of Table IV). The conditions of such precipitation were established by determining both yield and specific activity of precipitates obtained at various pH values and salt concentrations, and are illustrated in Figs. 5 and 6. The selection of the proper salt concentration for precipitation involved a compromise between yield and specific activity.

To 11 l. of solution that had passed through the column was added 7.4 l. of saturated ammonium sulfate solution. This brought the degree of saturation with respect to am-

TABLE IV

TOTAL ACTIVITY AND SPECIFIC ACTIVITY OF FRACTIONS OBTAINED AT VARIOUS STAGES OF THE PURIFICATION OF D-AMINO ACID OXIDASE

Stage of purification	Total activity, μl. O_2/10 min.	Specific activity, μl. O_2/10 min./mg. protein
Crude extracts	764,000[a]	6.4
Ppt. I First ammonium sulfate ppt.	1,200,000	640
Ppt. II Second ammonium sulfate ppt.	1,010,000	1390

[a] This figure must be doubled due to the presence of catalase in the crude extract. Catalase is not present in subsequent fractions.

monium sulfate to 0.4 and the *p*H to 7.0. The white precipitate which formed was collected by filtration after first adding 10 g. of Hyflo Super-Cel filter aid. The "Cel"-enzyme filter cake was extracted four times with 130-ml. portions of *M*/25 pyrophosphate buffer, *p*H 8.3, to give a final volume of 530 ml. of a clear yellow solution. This solution was brought to 0.3 saturation with respect to ammonium sulfate and the yellow precipitate which formed was collected by centrifugation at 317 × gravity for 20 min. The yellow precipitate was resuspended in distilled water to yield 100 ml. of dark amber solution. Data on the activity of the various fractions obtained in the course of this procedure are presented in Table IV.

Specific Property Test.—The procedure of Falconer and Taylor[6] was used. A graphic representation of the results is presented in Fig. 4.

Column Chromatography.—Hyflo Super-Cel was treated according to the procedure of Clauser.[12] Five grams of this treated material was added, as a slurry with the buffer (pyrophosphate *p*H 6.0) in which absorption was to take place, to a 7 × 350 mm. column. The column was washed with more of the same buffer and then 3.0 ml. of the enzyme containing 10 mg. of protein in buffer *p*H 6.0, added. The enzyme was eluted from the column by slowly raising the *p*H of the column.

A continuous *p*H gradient was maintained along the column by allowing a more alkaline pyrophosphate buffer, *p*H 8.3, to siphon into a well stirred acidic pyrophosphate buffer, *p*H 6.0, which in turn siphoned into the column. The rate of elution was adjusted to 5 ml./hr. An automatic fraction collector was used to collect the fractions. The whole procedure was conducted in a cold room at 0°. All of the protein (10 mg.) was recovered in 100 ml. of eluate.

Filter Paper Electrophoresis.—The apparatus was of the hang-strip modification built after the design of Kunkel.[13] The purified enzyme solution was added from a 15 *lambda* micropipet to inch wide strips of Whatman No. II paper. The application was made at the apex of the strip after dialyzing the enzyme against *M*/20 alanine buffer, *p*H 8.3 for four hours. A small drop of dextran was also placed at the apex in order to account for electroösmosis. Applied potentials ranged from 200 to 500 volts and times from 2–12 hours. At the completion of an experiment the position of the protein band was determined by staining with brom phenol blue in the manner deseribed by Durrum.[14] In addition several of these strips were segmented immediately after electrophoresis and activity determinations made on each segment. The position of maximum activity always corresponded to the position of the protein band within the limits of experimental error. The results of such an experiment are presented in Fig. 3.

Conventional Electrophoresis.—Purified enzyme solutions were first dialyzed overnight against the buffer in which the electrophoresis was to take place. Protein determinations made before and after dialysis were the same within experimental error. The positions of the boundaries were recorded photographically at appropriate intervals. Fractions were withdrawn from the cell at the end of the experiment by means of a syringe and needle. The activity and protein content of these fractions were determined in the usual manner. The results of these experiments are presented in Fig. 4 and Table III.

(12) H. Clauser and Choh Hao Li, THIS JOURNAL, **76**, 4337 (1954).

(13) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

(14) E. I. Durrum, THIS JOURNAL, **72**, 2943 (1950).

COLLEGE PARK, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Peptide Derivatives Containing Hydroxyamino Acids

BY JOHN C. SHEEHAN, MURRAY GOODMAN[1] AND GEORGE P. HESS[2]

RECEIVED SEPTEMBER 12, 1955

With the use of N,N′-dicyclohexylcarbodiimide as the condensing agent, several peptide derivatives containing L-serine, L-threonine and L-hydroxyproline have been synthesized.

In view of the outstanding importance of proteins and peptides containing hydroxyamino acids[3–8] and the complex problems associated with their synthesis, we have extended our recently devised method of peptide formation[9] to include derivatives of serine, threonine and hydroxyproline. Difficulties previously encountered have involved mainly interference by the hydroxyl group. Successful syntheses of these compounds have been accomplished only *via* the azide method, a multistep procedure.[10–14] With our technique, N,N′-dicyclohexylcarbodiimide[15] was used to form the peptide bond. Although the first aliphatic carbodiimide, diethyl carbodiimide, was prepared as early as 1893,[16] the reactions of these compounds with

(1) Aided by a contract from the Office of Naval Research.

(2) Aided by a fellowship from the National Foundation for Infantile Paralysis.

(3) N. K. Schaffer, S. C. May, Jr., and W. H. Summerson, *J. Biol. Chem.*, **202**, 67 (1953).

(4) N. K. Schaffer, S. Harshman, R. R. Engle and R. W. Drisco, *Fed. Proc.*, **14**, 275 (1955).

(5) M. Flavin, *J. Biol. Chem.*, **210**, 771 (1954).

(6) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951).

(7) P. H. Bell, *et al.*, THIS JOURNAL, **76**, 5565 (1954); P. H. Bell, *et al.*, *ibid.*, **77**, 3419 (1955).

(8) T. Wieland and W. Schön, *Ann.*, **593**, 157 (1955).

(9) J. C. Sheehan and G. P. Hess, THIS JOURNAL, **77**, 1067 (1955).

(10) J. S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942).

(11) D. W. Woolley, *ibid.*, **172**, 71 (1948).

(12) K. Hofmann and A. Jöhl, THIS JOURNAL, **77**, 2914 (1955).

(13) E. L. Smith and M. Bergmann, *J. Biol. Chem.*, **153**, 627 (1944).

(14) M. Brenner, K. Rüfenacht and E. Sailer, *Helv. Chim. Acta*, **34**, 2096 (1951).

(15) In reference 9 the wrong authors unfortunately were credited for the preparation of this reagent. The correct authors are E. Schmidt, F. Hitzler and E. Lahde, *Ber.*, **71**, 1933 (1938).

(16) F. Chancel, *Compt. rend.*, **116**, 330 (1893).