[CONTRIBUTION FROM THE INSTITUTE OF EXPERIMENTAL BIOLOGY, UNIVERSITY OF CALIFORNIA]

Iodination of Tyrosine Groups in Serum Albumin and Pepsin[1]

BY CHOH HAO LI

When protein is denatured, the presence of certain polar groups in the molecule becomes more evident. The behavior of the sulfhydryl group after denaturation has received much attention[1a] but the reactions of other groups in protein are not well known. The differences in the state of tyrosine in the native and denatured protein have been shown by the reaction with ferricyanide,[2] Folin phenol reagent[3] and porphyrindin[4] and by the changes in the absorption spectrum[5] with the alteration of the *p*H.

The reactions of iodine with proteins have received considerable attention. In the cases of zein,[6] insulin,[7] pepsin,[8] and lactogenic hormone,[9] iodine reacts only with the phenolic groups in the tyrosine residue of the protein. When the protein contains sulfhydryl groups, these groups take up iodine but they differ from tyrosine in that they react with iodine in acid solutions whereas tyrosine does not.[10] Moreover, tryptophan and histidine in proteins are inert in the conditions studied, though these amino acids when free[9,10,11] react with iodine. It appears, therefore, that iodine could be employed to study the reactivity of the tyrosine groups in proteins. This paper describes the results of such a study with pepsin and serum albumin. It will be seen that the behavior of the tyrosine groups in the native protein is different from that of these groups when the protein is in the denatured state.

The Reaction of Iodine with Native Serum Albumin.—The human serum albumin used was an amorphous preparation; its tyrosine content as determined by Lugg's method[12] was found to be 4.67%. This is in good agreement with the value reported by Brand, *et al.*[13] The fact that the protein did not take up iodine in acid solution indicates the absence of SH groups, confirming the findings of Greenstein.[14]

If the tyrosine in serum albumin is completely iodinated, it would require 5.16×10^{-4} mole of iodine per gram of protein. In an acetate buffer of *p*H 5.70, it was found that one gram of the protein absorbed only 2.10×10^{-4} mole of iodine after iodine treatment for sixty hours at room temperature. In order to sustain the concept that the sole action of iodine is with the tyrosine groups in the protein, we isolated the iodinated albumin and determined the iodine content by the method of Stimmel and McCullagh[15] and the free tyrosine content by the method of Lugg.[12] It was found that the iodinated protein contained 2.52% iodine and 2.80% free tyrosine. From the free tyrosine content, the theoretical iodine content is computed to be 2.67%. There is hence no indication that other groups in the protein react with iodine.

That the tyrosine groups in serum albumin cannot be completely iodinated in the acetate buffer is further demonstrated by following the rate of iodine disappearance. The solvent used was a *p*H 5.70 acetate buffer containing 0.182 *m* sodium acetate and 0.0341 *m* potassium iodide. The technique of rate measurements has been described previously.[16] Results are summarized in Fig. 1; curve A is obtained with serum albumin while B is obtained with free tyrosine. Each curve is plotted from data of duplicate experiments. The protein concentration was 12.5 mg. per cc. and the initial concentration of tyrosine 2.65×10^{-3} *m*. It is clear from Fig. 1 that the absorption of iodine in the protein solution is much slower in the later stage of the reaction though the initial rate is almost identical with that of free tyrosine.

Effect of Urea on the Iodination of Tyrosine.—Before we investigated the effect of urea on the reaction of iodine with protein, we studied the influence of this denaturing agent on diiodotyrosine formation. Table I summarizes the data indicating the catalytic effect of urea[16a] on the

TABLE I

EFFECT OF UREA ON THE IODINATION OF TYROSINE AT 25°

Solvent *p*H 5.70 acetate buffer, I^- 3.41×10^{-2} *m*; I_2 5.30×10^{-3} *m*; tyrosine 2.65×10^{-3} *m*

Urea, *m*	k_2 Found	k_2 Calcd. by eq. (2)
0	1.76	
1.0	2.10	2.14
4.0	2.57	2.52
7.1	2.75	2.78

(1) Aided by grants from the Board of Research of the University of California, and the Rockefeller Foundation, New York, N. Y.

(1a) Hans Neurath, J. P. Greenstein, F. W. Putman and J. O. Erickson, *Chem. Rev.*, **34**, 157 (1944).

(2) A. E. Mirsky and M. L. Anson, *J. Gen. Physiol.*, **19**, 451 (1936).

(3) (a) R. M. Herriott, *ibid.*, **21**, 501 (1938); (b) G. L. Miller, *J. Biol. Chem.*, **146**, 339 (1942).

(4) E. Brand and B. Kassell, *ibid.*, **138**, 437 (1940); **146**, 365 (1942).

(5) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(6) A. Neuberger, *ibid.*, **28**, 1982 (1934).

(7) C. R. Harrington and A. Neuberger, *ibid*, **30**, 810 (1936).

(8) R. M. Herriott, *J. Gen. Physiol.*, **20**, 335 (1937).

(9) C. H. Li, W. R. Lyons and H. M. Evans, *J. Biol. Chem.*, **138**, 43 (1941).

(10) M. L. Anson, *J. Gen. Physiol.*, **23**, 321 (1940).

(11) C. H. Li, THIS JOURNAL, **66**, 225 (1944).

(12) J. W. H. Lugg, *Biochem. J.*, **32**, 775 (1938).

(13) E. Brand, B. Kassell and J. L. Saidel, *J. Clin. Investigation*, **23**, 437 (1944).

(14) I. P. Greenstein, *J. Biol. Chem.*, **136**, 795 (1940).

(15) B. F. Stimmel and D. R. McCullagh, *ibid.*, **116**, 21 (1936).

(16) C. H. Li, THIS JOURNAL, **64**, 1147 (1942).

(16a) The *p*H of the acetate buffer in urea was adjusted to *p*H 5.70 after the addition of urea.

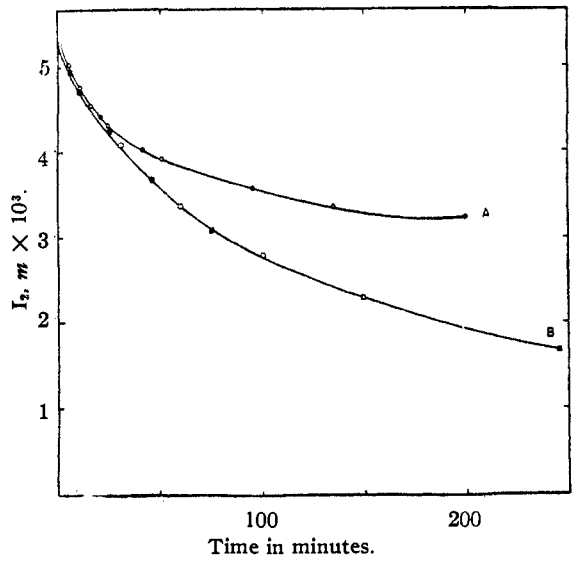


Fig. 1.—Rate of iodine disappearance in *p*H 5.70 acetate buffer solutions of serum albumin and tyrosine at 25°; protein concentration, 12.5 mg. per cc.; tyrosine concentration, 2.65×10^{-3} *m*; I^-, 3.41×10^{-2} *m*: Curve A, ○ ●, reaction with serum albumin; Curve B, □ ■, reaction with tyrosine.

specific rate of iodine–tyrosine reaction. It may be recalled that the diiodotyrosine formation follows a bimolecular reaction rate[16]

$$dx/dt = k_2(a - x)(b - 2x) \qquad (1)$$

where a and b are the initial concentrations of tyrosine and iodine, respectively, and x is that of diiodotyrosine. The specific rate, k_2, was calculated by the integrated form of equation (1). The concentrations are in moles per liter and time in minutes. In a previous paper[17] it has been demonstrated that the fact that the acetate and phosphate ions accelerate the iodination rate of tyrosine is due to the basic nature of these ions. The present results with urea may also be explained by the basic character of the catalytic agent. However, there is one special feature of the urea effect, *i. e.*, the increment of specific rate is not a linear function of urea concentration; instead, the following relationship is observed

$$k_2 = 1.76 + 0.38\sqrt{m} \qquad (2)$$

where m is the concentration of urea. The validity of equation (2) may be observed in Table I.

Iodination of Serum Albumin in Urea.—Since urea accelerates the rate of iodination of free tyrosine it would be expected that it also increases the rate of iodine up-take by serum albumin. In *p*H 5.70 acetate buffer containing 7.1 *m* urea, it was noted that iodine reacts with serum albumin at a faster rate than that in acetate buffer without urea. The rate is, however, still slower when it compares with the iodination rate of free tyrosine in the same solvent. If urea solutions of serum albumin were kept in 70° water-baths for some time and then treated with iodine, it was found that the protein, after ten minutes of heat treatment, absorbed iodine at a faster rate than did free tyrosine. When the heat treatment extended to thirty or sixty minutes, the rate of iodination became even greater. There was, however, no difference in rate between thirty- and sixty-minute treatments; the same iodination rate was also observed after the urea solution of serum albumin was put into 100° water-bath for ten minutes. These results are presented graphically in Fig. 2.

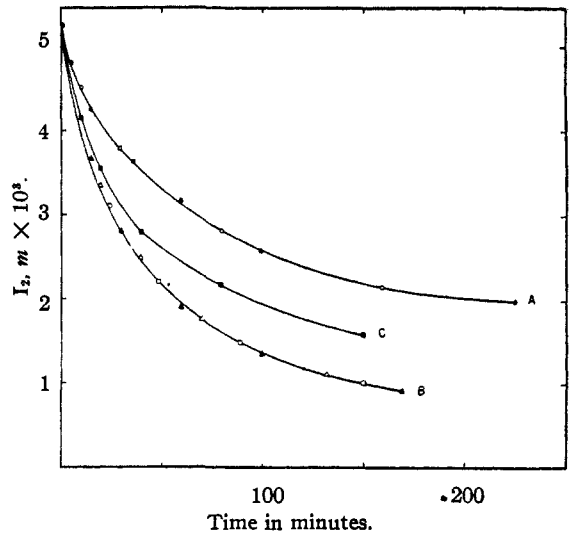


Fig. 2.—Rate of iodine disappearance in *p*H 5.70 acetate buffer–urea (7.1 *m*) solution of serum albumin at 25°, protein, 12.5 mg. per cc.; I^-, 3.41×10^{-2} *m*: Curve A, ○ ●, reaction without previous heat treatment; Curve B, △ ▲, reaction after treatment at 70° for thirty minutes or □ at 100° for ten minutes; Curve C, ■, reaction after treatment at 70° for ten minutes.

If we assume that all the phenolic groups in serum albumin are available for iodination after urea treatment at 70° for thirty minutes, and that all phenolic groups in the heat-urea denatured protein react with iodine as in free tyrosine, the computed bimolecular specific iodination rate should be constant. This is found to be the case; Table II presents typical kinetic data from an experiment using the protein which has been kept at 70° for thirty minutes in 7.1 *m* urea. The last column is the specific rate, k_2, calculated based upon the above assumptions. The initial tyrosine concentration was estimated from the tyrosine content in the protein. The averaged k_2 is shown to be 5.32 which is considerably higher than that for the free tyrosine (2.78, see Table I). This is in harmony with findings that peptide chains exert a catalytic effect on the iodination of phenols.[18]

Thus, the fact that the amount of iodine ab-

(17) C. H. Li, THIS JOURNAL, **66**, 228 (1944).

(18) C. H. Li, unpublished results.

TABLE II

IODINATION OF SERUM ALBUMIN IN UREA SOLUTION AFTER BEING KEPT AT 70° FOR TEN MINUTES

Solvent *p*H 5.70 acetate buffer in 7.1 *m* urea; I^-, 3.41×10^{-2} *m*; protein, 12.5 mg. per cc.

t, min.	I_2, *m* × 10^3	k_2
0	5.30	
5	4.60	5.25
15	3.70	5.26
30	2.82	5.62
60	1.92	5.54
100	1.38	5.10
170	0.92	5.17
		Av. 5.32

sorbed by serum albumin in acetate buffer is less than the theoretical quantity required by its tyrosine content may be explained by the fact that some of the tyrosine groups in the protein are not available for iodination. In the presence of urea, the denatured serum albumin liberates part of these unreactive tyrosine groups. As shown in Fig. 2, urea alone is not sufficient to liberate all these groups; a combination with heat denaturation is needed for this process. In order to sustain these interpretations, we have attempted to compute the number of free tyrosine groups in the native or partially denatured protein from the kinetic data.

The integrated form of equation (1) may be written

$$k_2 t = \frac{1}{b - 2a} \ln \frac{a(b - 2x)}{b(a - x)} \qquad (3)$$

which can be rearranged

$$\frac{a(b - 2x)}{b(a - x)} = e^{[k_2 t(b - 2a)]} \qquad (4)$$

By first approximation equation (4) becomes

$$a(b - 2x) = b(a - x)[1 + k_2 t(b - 2a)]$$

This is simplified to the form

$$a = x + \frac{x}{k_2 t b} \qquad (5)$$

Hence, if the specific rate, k_2, is known, the initial concentration of tyrosine, a, could be computed in a single kinetic experiment.

As shown in Table II, the specific rate for the iodination of completely denatured serum albumin is 5.32, which may be employed as k_2 in equation (5) for the calculation of a in urea solutions of serum albumin. Table III illustrates the usefulness of equation (5) and the validity of our assumption; the calculated values of a are averaged to be 1.73×10^{-3} *m*. If the tyrosine residue in the protein is all available for the reaction, its concentration should be 2.65×10^{-3} *m*. Therefore it may be said that in 7.1 *m* urea, 33% of the tyrosine groups in serum albumin are not free to react with iodine. Taking the molecular weight[19] of serum albumin as 72,000, we assume that serum albumin contains 18 tyrosine groups per molecule and that six of these groups are situated in such a position that they cannot be attacked by iodine.

(19) J. L. Oncley, quoted by E. J. Cohn, *Trans. Studies College of Physicians of Philadelphia*, **10**, 149 (1942).

TABLE III

THE CALCULATED CONCENTRATION OF FREE TYROSINE IN 7.1 *m* UREA SOLUTION OF SERUM ALBUMIN

Protein, 12.5 mg./cc.; I^- 3.41×10^{-2} *m*; solvent *p*H 5.70 acetate buffer in 7.1 *m* urea.

t, min.	I_2, *m* × 10^3	*x*, *m* × 10^3	*a*,[b] *m* × 10^3
0	5.30		
5	4.85	0.225	1.82
15	4.30	.500	1.68
36	3.65	.920	1.64
60	3.20	1.050	1.67
100	2.60	1.350	1.83
			Av. 1.73

[b] *a* calculated by equation (5) taking $k_2 = 5.32$.

As shown in Fig. 2, the tyrosine groups in serum albumin are not completely liberated when the urea solution is held at 70° for ten minutes. We have also calculated the concentration of the available tyrosine in this urea-heat denatured, a, by equation (5) taking k_2 as 5.32, and found to be 2.31×10^{-3} *m*. Thus, 13% of the tyrosine groups in this urea-heat denatured protein are not available for the iodination, *i. e.*, two of these groups per protein molecule are still inert.

For the estimation of the concentration of free tyrosine groups in the native serum albumin, we have no means to determine k_2 in order that equation (5) can be utilized. We have reason, however, to believe that the value of k_2 cannot be greater than that in urea solution, and less than that for free tyrosine in acetate buffer. We have, therefore, chosen k_2 to be 4.5 and found that $a = 1.29 \times 10^{-3}$ *m*. This suggests that 50% of the phenolic groups in the native serum albumin or nine tyrosine groups per molecule are available for iodination.

The State of Tyrosine in Pepsin.—A commercial preparation of crystalline pepsin was employed; the protein solution was thoroughly dialyzed against distilled water and then lyophilized.

It has been shown[20] that pepsin contains no SH groups and has a high tyrosine content. As has already been mentioned, iodine reacts only with the tyrosine groups in pepsin.[3a] The claim of Philpot and Small,[21] that some group other than tyrosine was involved, has recently been refuted by Herriott.[22]

Crystalline pepsin contains 7.88% tyrosine as determined by the method of Lugg.[12] The fact that it did not react with iodine in acid solution indicates the absence of SH groups. In *p*H 5.70 acetate buffer, the disappearance of iodine was measurable; curve A in Fig. 3 gives the course of

(20) H. O. Calvery, R. M. Herriott and J. H. Northrop, *J. Biol. Chem.*, **113**, 11 (1938).

(21) J. St. L. Philpot and P. A. Small, *Biochem. J.*, **33**, 1727 (1939).

(22) R. M. Herriott, *J. Gen. Physiol*, **25**, 185 (1941).

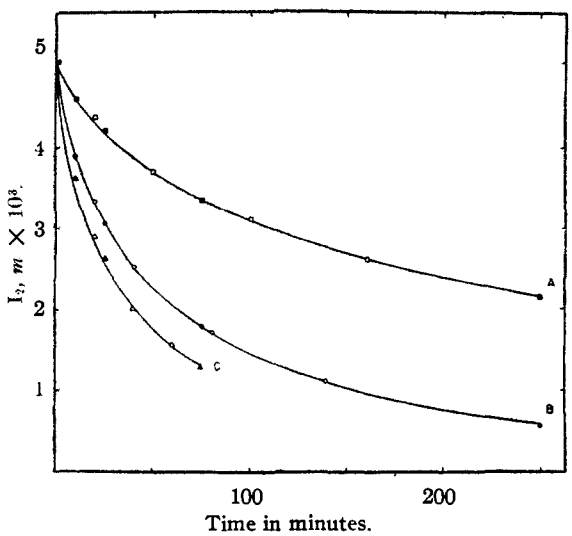


Fig. 3.—Rate of iodine disappearance in acetate buffer and in urea solutions of pepsin at 25°: protein concentration, 5.90 mg. per cc.; I^-, 3.42 × 10^{-2} *m*: Curve A, □ ■, reaction in *p*H 5.70 acetate buffer; Curve B, ○ ●, reaction in *p*H 5.70 acetate buffer–urea (7.1 *m*) solution; Curve C, △ ▲, reaction in *p*H 5.70 acetate buffer–urea (7.1 *m*) solution, after the solution is kept at 70° for thirty minutes.

iodine uptake by pepsin in this buffer. The protein concentration was 5.90 mg. per cc.; other conditions were the same as in the serum albumin experiments. The rate of iodine disappearance is very similar to that for free tyrosine (*cf.* Fig. 1) though it is somewhat slower. No good specific rate constant was obtained when either monoiodotyrosine or di-iodotyrosine formation was assumed. As in the case of serum albumin, explanation may reside in the fact that not all the tyrosine groups in pepsin are available for the reaction.

When pepsin was dissolved in 7.1 *m* urea, it absorbed iodine more rapidly. If the protein in urea solution had been kept at 70° for thirty minutes, the rate of reaction was even greater. Results of these experiments show graphically in Fig. 3; each curve is drawn from the data of duplicate experiments. Inspection of these curves shows that curve B cannot be represented as the expected data for either di-iodotyrosine or monoiodotyrosine formation on the assumption that all phenolic groups are exposed for iodination, whereas based upon this assumption, curve C gives a good specific rate for di-iodotyrosine formation. This is evident in the results shown in Table IV. It is therefore reasonable to conclude that, as in serum albumin, all the phenolic groups in urea-heat denatured pepsin are as free to react with iodine as in free tyrosine and that all or nearly all of the tyrosine in the protein is converted into di-iodotyrosine.

Following the method employed for the serum albumin experiments, we have computed the concentration of reactive tyrosine groups in native

TABLE IV

IODINATION OF CRYSTALLINE PEPSIN IN UREA SOLUTION AFTER BEING KEPT AT 70° FOR THIRTY MINUTES

Protein 5.90 mg. per cc.; I^- 3.41 × 10^{-2} *m*; solvent *p*H 5.70 acetate buffer in 7.1 *m* urea

t, min.	I_2, *m* × 10^3	k_2
0	5.10	
10	3.66	7.71
20	2.88	7.40
40	2.02	7.39
60	1.55	7.50
		Av. 7.50

or urea-denatured pepsin by equation (5) taking k_2 as 7.50 (see Table IV); it was found that 2.18 × 10^{-3} *m* of tyrosine out of 2.55 × 10^{-3} *m* in the protein is available for iodination. Similarly, assuming k_2 = 3.0 in acetate buffer, the concentration of reactive tyrosine groups in native pepsin is computed to be 1.73 × 10^{-3} *m*, *i. e.*, 68% of tyrosine groups in native pepsin is reactive. Thus, from the molecular weight[23] and tyrosine content of pepsin, the number of free tyrosine groups in the native protein is computed to be 12 out of 17 groups, while three of the five "unavailable" groups are liberated in 7.1 *m* urea solutions.

The enzymatic activity of pepsin has been shown to be unimpaired by three hours of treatment with 4 *m* urea.[24] From the present study it is evident that the pepsin molecule is definitely altered in urea solutions. Now, the activity of pepsin decreases as the amount of iodine absorbed by the protein increases.[8] It may then be said that the steric arrangement of the tyrosine groups in pepsin is not essential for the proteolytic activity, though modifications[8,25] of these groups destroy the activity.

Proteins such as pepsin and serum albumin possess a nearly spherical shape, indicating an arrangement of peptide chains in a condensed and oriented configuration. The denaturation of protein in urea solutions has thus been described as a process of unfolding of these peptide chains. It is likely that part of the tyrosine residue in the native protein is hidden inside the molecule and that only surface phenolic groups react with iodine. When the folded peptide chains are opened by a denaturing agent, the concealed tyrosine groups become accessible.

Summary

(1) The fact that iodine reacts only with the tyrosine residues in human serum albumin has been demonstrated. (2) It is shown that urea accelerates the rate of diiodotyrosine formation. (3) The rate of iodinating serum albumin and pepsin has been investigated in acetate buffer and in urea solution. In acetate buffer, the tyrosine groups are not all reactive with iodine; some but

(23) I. B. Erickson-Quensel, unpublished [*cf.* T. Svedberg and I. B. Erickson-Quensel, *Tab. Biol.*, 11, 361 (1936)].

(24) J. Steinhardt, *J. Biol. Chem.*, **123**, 543 (1938).

(25) R. M. Herriott, *J. Gen. Physiol.*, **19**, 283 (1938).

not all of these unreactive groups can be liberated in the presence of urea. When both heat and urea are used as denaturing agents, all tyrosine groups become available for iodination. (4) From the kinetic data, a computation has been made of the number of free tyrosine groups in pepsin and in serum albumin.

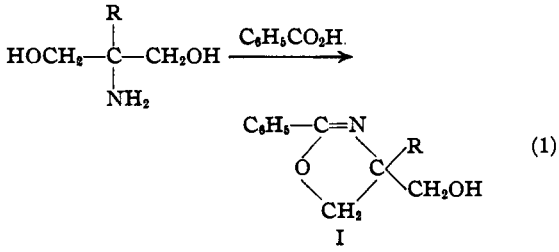
BERKELEY, CALIFORNIA RECEIVED FEBRUARY 12, 1945

[CONTRIBUTION OF THE CHEMICAL LABORATORY OF INDIANA UNIVERSITY]

Amino Acids. IV. Amino Acids Related to Serine

BY JOHN H. BILLMAN AND EARL E. PARKER

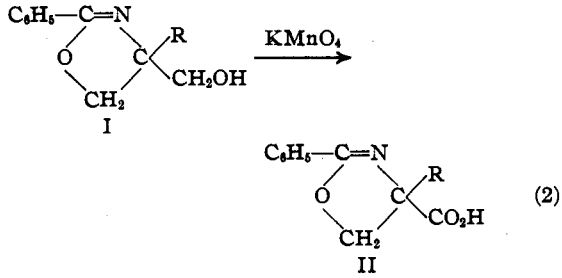
A new method[1] for synthesizing amino acids has now been extended to include the conversion of dihydric and trihydric amino alcohols to β-hydroxy-α-amino acids. The amino alcohols were first allowed to react with benzoic acid in xylene to yield the corresponding oxazolines (I).



(1)

The group R stands for $-CH_3$, $-C_2H_5$, or $-CH_2OH$.

The oxazolines were then oxidized to the corresponding oxazoline carboxylic acids II with alkaline permanganate.



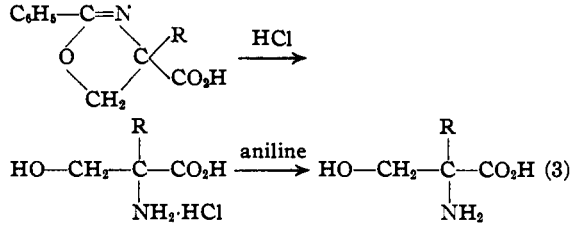
(2)

R stands for $-CH_3$, $-C_2H_5$, or $-COOH$.

When R was ethyl or methyl, the acid II proved to be difficult to isolate and for this reason the mixtures were hydrolyzed without isolating the carboxylic acids. When R was —COOH the dibasic acid was isolated in the pure state but again it was more convenient to hydrolyze the entire oxidation mixture.

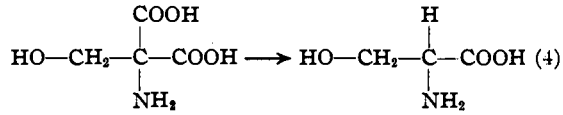
The oxazolines were hydrolyzed with hydrochloric acid to the corresponding β-hydroxy-α-amino acid hydrochloride and benzoic acid. Treatment of these amino acid hydrochlorides with aniline produced β-hydroxy-α-amino acids that were free of halogen.

(1) Billman and Parker, THIS JOURNAL, 66, 538 (1944).



(3)

In the case when R = COOH it was hoped that this compound could be decarboxylated to yield serine.



(4)

Numerous attempts to decarboxylate this compound or its hydrochloride failed to produce pure serine.

Likewise 2-phenyl-Δ²-oxazoline-4,4-dicarboxylic acid did not decarboxylate satisfactorily. However, further attempts are being made to prepare pure serine.

Experimental

2-Phenyl-4-methyl-4-hydroxymethyl-Δ²-oxazoline.—In a 3-liter round-bottomed flask were placed 1500 cc. of xylene, 219.6 g. (1.8 moles) of benzoic acid, and 189 g. (1.8 moles, m. p. 105–107°) of 2-amino-2-methyl-1,3-propanediol. The flask was equipped with a moisture trap and a water condenser. The contents were refluxed by means of an electrically heated oil-bath maintained at 170–180° until approximately the theoretical amount of water was collected. The time required for this reaction was ninety-six hours while a 0.3 mole reaction required only twenty-four hours.

The reaction product crystallized on being chilled in the refrigerator. The solid was filtered off and the filtrate was concentrated to 750 cc. and then to 150 cc. to obtain additional amounts of crude material. When 100 g. of this material was recrystallized from a solution containing 120 cc. of 95% alcohol and 240 cc. of water 83.5–86.5 g. of pure product melting at 103–104° was obtained; yield 67–69%. *Anal.* Calcd. for $C_{11}H_{13}O_2N$: N, 7.33. Found: N, 7.43.

α-Methylserine.—In a two-liter 3-necked flask were placed 900 cc. of water, 8 g. of sodium hydroxide, and 57.3 g. of pulverized 2-phenyl-4-methyl-4-hydroxymethyl-Δ²-oxazoline. To this vigorously stirred mixture was added 63 g. of potassium permanganate in small portions at such a rate that the temperature never exceeded 40°. The reaction was complete in ninety minutes. The manganese dioxide was filtered off and the filtrate acidified with hydrochloric acid and the mixture refluxed nine hours.