b. p. 280–282° (13 mm.), recrystallized from benzene-petroleum ether, m. p. 114°; yield 38.2 g. or 66% based on the 8-amino-6-methylquinoline (0.3 mole) taken.

Anal. Calcd. for $C_{13}H_{10}N_2$: C, 80.38; H, 5.19; N, 14.43. Found: C, 80.58; H, 5.28; N, 14.60.

5(6)-Methyl-(1,10)-phenanthroline Monopicrate.—Fine yellow needles from 95% ethanol; m. p. 203–204°.

Anal. Calcd. for $C_{19}H_{13}N_5O_7$: C, 53.89; H, 3.10; N, 16.54. Found: C, 53.96; H, 3.27; N, 16.67.

5-Nitro-6-methyl-(1,10)-phenanthroline.—A mixture of 1.00 g. of 5(6)-methyl-(1,10)-phenanthroline (m. p. 114°), 5 cc. of sulfuric acid (sp. gr. 1.84) and 3 cc. of nitric acid (sp. gr. 1.42) was held at 120° for two hours. The yellow nitration mixture was poured onto 50 g. of ice and the cold solution neutralized with 30% sodium hydroxide. The precipitate was filtered off and dried: fine yellow needles from 95% ethanol; m. p. 268–270°; yield 0.98 g. (80%).

Anal. Calcd. for $C_{13}H_9N_3O_2$: N, 17.57. Found: N, 17.69.

Acknowledgment.—The authors gratefully acknowledge the valuable suggestions offered by Professor C. S. Marvel in the course of this work.

Summary

1. A procedure for the synthesis of 5(6) derivatives of 1,10-phenanthroline has been described.

2. The previously reported synthesis of 5(6)-bromo-1,10-phenanthroline has been proved erroneous.

3. Evidence for the equivalence of the 5- and 6-positions in 1,10-phenanthroline has been presented. This in turn indicates a symmetrical structure for 1,10-phenanthroline.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, MEDICAL RESEARCH DIVISION, SHARP & DOHME, INC.]

Tryptic Digestion of Bovine Serum and Other Proteins in the Presence of Ethyl Alcohol

BY EDWIN A. RISLEY, ANN C. BUFFINGTON AND L. EARLE ARNOW

In connection with some problems under investigation in this Laboratory, it became desirable to prepare tryptic digests of bovine serum. The chief difficulties encountered revolved around the slow digestion of relatively concentrated serum solutions and the development of putrefaction after a few days of digestion. It was found that both of these difficulties could be overcome if suitable amounts of ethyl alcohol were included in the digestive mixtures.

The effect of alcohol on tryptic digestion has not been investigated extensively. In 1896 Chittenden and Mendel[1] found that the digestion of fibrin by pancreatic extracts was inhibited by increasing concentrations of alcohol. However, appreciable digestion still occurred in 20% alcoholic solution. Vernon[2] stated that extracts of pancreas prepared with 25% alcohol were slightly less active in digesting fibrin than were similar extracts prepared with saline solution. Gizelt,[3] using egg white in Mett tubes as a substrate and pancreatic juice as a source of enzyme, found that alcohol in concentrations less than 20% inhibited enzymatic activity. Concentrations of 20% or higher abolished all enzymatic activity. Bayliss[4] suspended crude trypsin in 80% alcohol. This suspension was reported to digest gliadin slowly. Since most of the tryptic activity could be removed from the suspension by simple filtration, Bayliss concluded that trypsin in an insoluble form must still be active. Edie[5] concluded that the action of crude trypsin on fibrin was inhibited to some degree by concentrations of alcohol higher than 3%. Some digestion took place in 25% alcohol, but none was detected at 50%. On the other hand, no appreciable inhibition of digestion of casein was noted until the alcohol concentration was raised to 10% and, indeed, digestion in 25% alcohol was almost as efficient as in non-alcoholic media. Some digestion of casein occurred even in the presence of 50% alcohol. Abderhalden and Reich[6] studied the influence of various alcohols on the rate of tryptic digestion of *d,l*-leucylglycyl-*d,l*-leucine. No significant inhibition of digestion in concentrations of ethyl alcohol of 10% or lower was detected. Twenty per cent. alcohol markedly inhibited digestion of the peptide.

Experimental

The bovine serum used in these experiments was supplied to us in spray dried form through the courtesy of Dr. David Klein of the Wilson Laboratories. Difco trypsin, 1:250, was used as a source of enzyme. The alcohol employed was 95% ethyl alcohol. A micro-Kjeldahl method was used for nitrogen determinations. Amino nitrogen determinations were done with the Koch[7] modification of the Van Slyke[8] micro-apparatus.

Van Slyke[9] pointed out many years ago that the presence of alcohol causes the formation of excess gas in the amino nitrogen apparatus. Provided the concentration of alcohol is not too high, the excess gas can be removed by increasing the shaking time during absorption. In all the analyses reported in this paper, the solutions were diluted and, in the great majority of instances, contained approxi-

(1) R. H. Chittenden and L. B. Mendel, *Am. J. Med. Sci.*, **111**, 181 (1896).
(2) H. M. Vernon, *J. Physiol.*, **26**, 405 (1901).
(3) A. Gizelt, *Arch. ges. Physiol.*, **111**, 620 (1906).
(4) W. M. Bayliss, *J. Physiol.*, **50**, 85 (1915).
(5) E. S. Edie, *Biochem. J.*, **13**, 219 (1919).
(6) E. Abderhalden and F. Reich, *Fermentforsch.*, **11**, 64 (1929).
(7) F. C. Koch, *J. Biol. Chem.*, **84**, 601 (1929).
(8) D. D. Van Slyke, *ibid.*, **16**, 121 (1913).
(9) D. D. Van Slyke, *ibid.*, **12**, 275 (1912).

mately 2% alcohol. In a few cases, the alcohol concentration in the diluted solution was as high as 10%. The following experiment indicates that analyses can be carried out accurately at these concentrations of alcohol. A tryptic digest of beef serum was diluted with water and with suitable alcohol water mixtures, and the various solutions thus formed were analyzed for total and amino nitrogen. The data are given in Table I.

TABLE I

ANALYSIS OF A DILUTED SERUM DIGEST IN THE PRESENCE AND IN THE ABSENCE OF ALCOHOL

Final alcohol concn., %		0	10	20
Nitrogen, mg. per cc.	Total	1.23	1.23	1.23
	Amino	0.32	0.32	0.33

Comparative Digestion of Bovine Serum and Casein in 20% Solution.—The following digestive mixtures were prepared: (1) 5 g. of bovine serum and 120 mg. of trypsin were suspended in sufficient 1% methyl *p*-hydroxybenzoate solution to make a volume of 25 cc. The mixture was well stirred. The methyl ester served as a preservative, although it was found to be ineffective for digestion periods of more than a few days. (2) 5 g. of serum, 120 mg. of trypsin, 5 cc. of alcohol, and sufficient water to make 25 cc. were mixed and well stirred. (3) Mixtures identical with the above two, except that casein was substituted for the dry serum, were prepared. All four mixtures were incubated at 37°. At the times indicated in Table II, aliquots were filtered and the filtrates were analyzed for total and amino nitrogen. The results obtained are given in Table II.

TABLE II

COMPARATIVE DIGESTION OF BOVINE SERUM AND CASEIN BY TRYPSIN

Time in hours	0[a]		24		48	
Substrate	TN[b]	AN%[c]	TN	AN%	TN	AN%
Bovine serum	12.5	7	23.0	16	21.0	26
Bovine serum + alcohol	10.6	10	20.1	30	24.1	32
Casein	0.7	4	22.7	39	23.2	44
Casein + alcohol	0.6	4	26.2	33	26.6	39

[a] Filtered at room temperature. [b] TN represents total nitrogen in mg. per cc. of filtrate. [c] AN% represents amino nitrogen as % of total nitrogen.

Under these experimental conditions, it is apparent that digestion of the beef serum proceeds more rapidly in the presence of the alcohol than in its absence. This difference in rate is most striking at twenty-four hours. The absolute values for amino nitrogen do not differ significantly in the presence or absence of alcohol when casein is the substrate, although somewhat more nitrogen goes into solution in the mixture containing alcohol.

Effect of Concentration of Trypsin on Digestion of Serum in the Presence of Alcohol.—Ten grams of dried serum was dissolved in 150 cc. of water with rapid stirring. After solution was complete, an additional 10 cc. of water was added. This serum solution was divided into 4 aliquots of 40 cc. each. To each aliquot were added 10 cc. of alcohol and the quantity of trypsin indicated in Table III. After stirring, all four mixtures were placed in the incubator at 37°. At various times, samples were removed and filtered, and the filtrates were analyzed for total and amino nitrogen. The results obtained are given in Table III.

As might have been anticipated, these data indicate that both the rate and extent of hydrolysis increase as the concentration of trypsin is increased. Incubation of serum with alcohol, but without added trypsin, resulted in precipitation of material containing nitrogen. The soluble material remaining after ninety-six hours of incubation was rich in amino nitrogen as compared with the original serum,

TABLE III

TRYPTIC DIGESTION OF BOVINE SERUM IN 20% ALCOHOLIC SOLUTION

Hours	Mg. of trypsin: 0		50		100		150	
	TN[a]	AN%[a]	TN	AN%	TN	AN%	TN	AN%
0	5.8	6	5.9	8	6.2	9	5.9	12
2	5.7	8	6.0	20	6.2	17	6.5	20
22	5.7	7	5.5	25	6.2	33	6.4	45
96	3.6	17	6.0	39	6.0	53	6.3	59
119	3.5	19	6.0	39	6.0	52	5.9	61

[a] See footnotes after Table II.

and it is apparent that some slight digestion occurred. Probably this was due to the presence of enzymes in the serum.

Effect of Concentration of Alcohol on Digestion of Serum.—Mixtures containing 5% serum, 0.3% trypsin, and varying concentrations of alcohol were prepared and incubated at 37°. Total and amino nitrogen determinations were made on filtrates at intervals. The results are given in Table IV.

The data in this table indicate that the rate and extent of hydrolysis were decreased in the presence of alcohol concentrations exceeding 30%, although appreciable hydrolysis occurred even in the presence of 60% alcohol. Digestion proceeded steadily for long periods of time in concentrations of 10 to 30% alcohol. Five per cent. alcohol was not sufficient to prevent the development of putrefaction.

Digestion of Various Proteins in the Presence of Alcohol.—In addition to the use of alcohol, we have also frequently utilized digestion at 60° as a means of preventing putrefaction. Comparative figures for tryptic digestion of several proteins, with and without 20% alcohol, and at 37 and 60°, are given in Table V. The digestive mixtures employed contained 5% protein and 0.3% trypsin, and were adjusted to *p*H 7.6 (the *p*H of the serum solution). Where alcohol was not present, 1% methyl *p*-hydroxybenzoate was added as a preservative.

At the intervals indicated in Table V, filtered samples of each mixture were analyzed for total and amino nitrogen. The figures for one hundred forty-eight hours cannot be compared directly with the other data, since they were obtained in a separate experiment carried out at a later date.

These data indicate that, in general, digestion at 37° is as efficient in the presence of alcohol as in its absence except in the case of serum during early phases of digestion. In the case of soy bean protein considerably more nitrogen went into solution when alcohol was present. When the digestion was allowed to proceed at 60°, the presence of the alcohol decreased the rate and extent of digestion of all the proteins tested. This effect was most marked in the case of egg albumin.

Discussion

The experimental data recorded in this paper make it evident that efficient tryptic digestion at 37° can be obtained in the presence of ethyl alcohol in concentrations of 30% or less. When 5% dried serum in 8 *N* sulfuric acid was heated under reflux for twenty-four hours, the soluble amino nitrogen was found to be 82% of the total soluble nitrogen. Under the conditions used in our experiments, then, 70 to 80% of the potential amino groups were liberated after one or two weeks of incubation with a single charge of trypsin.

Putrefaction was not observed when the concentration of alcohol was 10% or higher. It would appear that alcohol should be a useful reagent for preventing putrefaction during the lengthy

TABLE IV

TRYPTIC DIGESTION OF BOVINE SERUM IN THE PRESENCE OF VARYING CONCENTRATIONS OF ALCOHOL

Hours	% Ethyl alcohol: 5 TN[a]	5 AN%[a]	10 TN	10 AN%	20 TN	20 AN%	30 TN	30 AN%	40 TN	40 AN%	50 TN	50 AN%	60 TN	60 AN%
0	5.3	13	5.8	13	6.0	13	5.9	14	3.1	15	3.0	19	1.0	20
24	5.6	43	5.7	37	6.0	39	5.5	43	4.1	38	3.8	35	2.4	30
47			5.7	46	6.2	46	5.5	53						
71			5.7	52	6.2	51	5.5	58						
96	5.7	59							4.8	43	3.6	40	2.9	36
119	[b]		5.6	55	6.2	52	5.6	58						
144									4.8	46	3.9	44	3.1	39
172									4.8	47	3.9	45	3.0	39
191			5.6	63	6.2	54	5.6	58						
220									4.8	48	4.0	46	3.1	46
263			5.9	63	5.9	57	5.5	60						

[a] See footnotes after Table II. [b] After ninety-six hours this sample developed an odor indicative of putrefaction, and the experiment was terminated.

TABLE V

EFFECT OF ALCOHOL (20%) ON TRYPTIC DIGESTION OF VARIOUS PROTEINS

Substrate	37°C. Time, hours: 24 TN[a]	24 AN%[a]	48 TN	48 AN%	148 TN	148 AN%	60°C. Time, hours: 24 TN	24 AN%	48 TN	48 AN%	148 TN	148 AN%
Bovine serum	6.4	43	6.9	55	6.6	57	6.4	49	6.6	51	6.3	61
Bovine serum + alcohol	6.1	53	6.0	59	6.5	62	5.8	36	5.8	38	5.1	53
Casein	7.1	54	7.3	59	7.0	56	6.9	44	7.4	53	6.9	49
Casein + alcohol	4.6	51	5.9	58	7.5	54	6.2	40	7.2	41	6.9	47
Soy bean protein	2.5	52	2.4	60	2.7	71	4.0	41	4.5	41	4.8	44
Soy bean protein + alcohol	3.1	47	3.6	55	4.3	62	2.9	38	3.4	39	2.9	44
Lactalbumin	5.6	59	5.6	65	5.6	75	5.7	57	5.8	60	5.6	54
Lactalbumin + alcohol	5.4	61	5.6	67	6.2	55	5.4	41	5.7	43	..	..
Egg albumin	6.4	55	6.7	60	6.2	68	6.7	57	6.9	58	6.4	60
Egg albumin + alcohol	5.6	48	6.4	54	6.6	62	2.6	56	3.0	57	2.6	72

[a] See footnotes after Table II.

digestion periods required for certain experimental procedures and for the commercial preparation of some types of peptone.

It has been known for years that an antitrypsin is present in blood sera. A purified preparation of this material has been described by Schmitz.[10] The same worker has suggested that the trypsin (or trypsin-like protease) normally present in plasma is combined with its antiprotease.[11] The data in Table II indicate that concentrated (*i. e.*, 20%) solutions of dried bovine serum are resistant to tryptic digestion in the absence of alcohol. It is possible that this resistance is caused by the presence of serum antiproteases. If this is true, the role of the alcohol would appear to be the inactivation of (or the release of trypsin from) these antiproteases. This explanation of the action of the alcohol in our experiments is made more probable by reports indicating that the antitryptic power of serum is removed by treatment with acetone, phenol or chloroform.[12]

The probable importance of serum proteases and antiproteases both in physiological and pathological processes has become increasingly evident in recent years. Much of the important work in this field has been reviewed by Grob.[13] The possible roles of serum antiprotease and protease in the blood-clotting mechanism have been stressed by Ferguson.[14] Marked changes in the antitryptic power of the blood associated with anaphylaxis were observed by Burdon.[15] There is good evidence that serum antiproteases inhibit the growth of bacteria.[16] Holmes, Keefer and Myers[16] have made the interesting suggestion that the antiproteases in body fluids are of importance in the prevention of damage to tissue by proteases liberated from the leukocytes that accumulate in regions of suppuration. It is possible that some of the beneficial effects produced by the parenteral administration of blood, plasma, and serum may be related to the proteases and antiproteases present in these fluids.

Summary

1. Concentrated (20%) solutions of dried bovine serum are resistant to the action of trypsin.

(10) A. Schmitz, *Z. physiol. Chem.*, **255**, 234 (1938).

(11) A. Schmitz, *ibid.*, **250**, 37 (1937).

(12) K. Okubo, *Tohôku J. Exptl. Med.*, **4**, 427, 441 (1924); *Chem. Abs.*, **18**, 2533 (1924).

(13) D. Grob, *J. Gen. Physiol.*, **26**, 405, 423, 431 (1943).

(14) J. H. Ferguson, *Science*, **97**, 319 (1943); *Proc. Soc. Exptl. Biol. Med.*, **51**, 373 (1942).

(15) K. L. Burdon, *ibid.*, **49**, 24 (1942).

(16) W. F. Holmes, C. S. Keefer and W. K. Myers, *J. Clin. Investig.*, **14**, 131 (1935).

This resistance can be overcome by the use of digestion mixtures containing 20% alcohol. It is suggested that the effect of the alcohol may be due to an inhibition of antiproteases present in the serum.

2. Tryptic digestion of serum proceeded smoothly in concentrations of 10 to 30% alcohol for as long as two weeks, at which time approximately 70 to 80% of the potential amino groups had been liberated.

3. Concentrations of alcohol higher than 30% inhibited tryptic digestion of serum, but appreciable hydrolysis occurred even in 60% alcohol.

4. Efficient digestion of several other proteins (casein, lactalbumin, soy bean protein, egg albumin) was obtained in the presence of alcohol (20%).

5. Digestion at 60°, instead of the more usual 37°, was less rapid and complete when alcohol (20%) was present in the digestion mixture.

6. It is suggested that ethyl alcohol is a useful reagent for preventing putrefaction during tryptic digestion.

GLENOLDEN, PA. RECEIVED OCTOBER 30, 1943

[CONTRIBUTION FROM THE AVERY LABORATORY OF CHEMISTRY OF THE UNIVERSITY OF NEBRASKA]

α,β-Diamino Ketones. II.[1] Reactions of Thalline and Open Chain Secondary Amines with α-Bromo-β-aminoketones

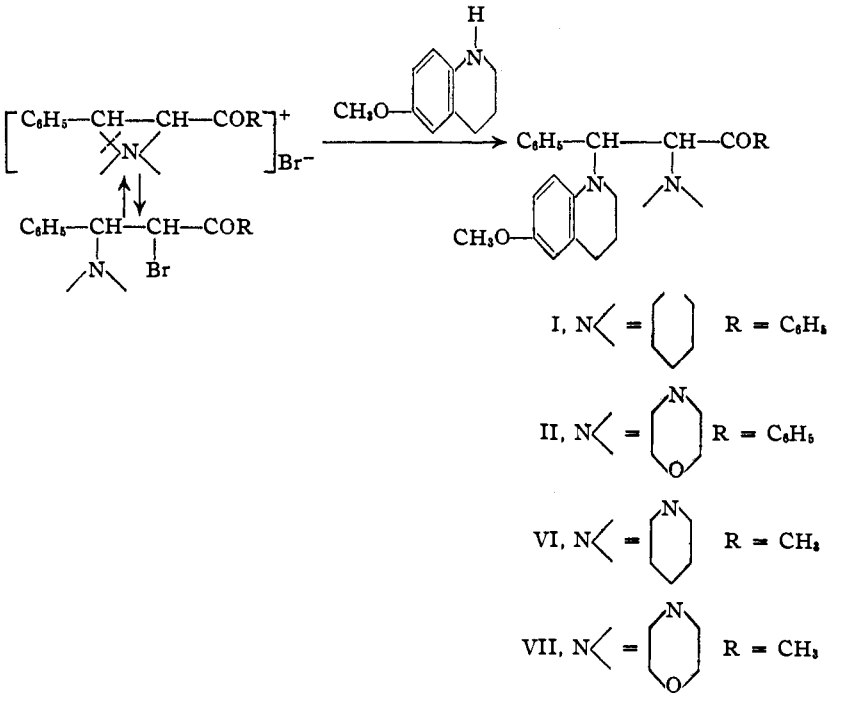
BY NORMAN H. CROMWELL, JOHN A. CAUGHLAN[2] AND GORDON F. GILBERT

A wide variety of mixed diamino ketones may be prepared from α-bromo-α,β-unsaturated ketones. Since tetrahydroquinoline had been shown to react with α-bromo-β-aminoketones to give excellent yields of mixed diamino ketones[3] it seemed important to prepare for chemotherapeutic investigations the analogous tetrahydro-6-methoxyquinolino (thallino) compounds. Thalline for these investigations was prepared by a high pressure reduction of 6-methoxyquinoline using hydrogen and a copper chromite catalyst. The 6-methoxyquinoline was prepared by a modified Skraup reaction.

β-Piperidino- and β-morpholino - α - bromobenzylacetophenone and the corresponding benzylacetones reacted readily with thalline to give good yields of the mixed diamino ketones.

The only diamino ketone which had been prepared by treating an α-bromo-β-amino ketone with an open chain type of secondary amine was α,β-di-N-methylbenzylaminobenzylacetophenone.[4] Since this product was obtained in such low yields it seemed of interest to establish whether or not such low yields are to be expected generally when an open chain secondary amine is used in these reactions.

It was found that β-piperidino-α-bromobenzylacetophenone and β-piperidino-α-bromobenzylacetone reacted with N-methylbenzylamine, di-



benzylamine and N-methylethanolamine[5] to give from fair to poor yields, of the mixed diamino ketones.

It has been shown previously[3b] that these reactions give the best yields of the mixed diamino

(1) For the previous paper in this series see: Cromwell, Harris and Cram, THIS JOURNAL, 66, 134 (1944).

(2) Eastman Kodak Co. Fellow, 1942–1943.

(3) (a) Cromwell, THIS JOURNAL, 63, 2984 (1941); (b) Cromwell and Cram, *ibid.*, 65, 301 (1943).

(4) Cromwell and Witt, *ibid.*, 65, 308 (1943).

(5) The N-methylethanol amine for these experiments was generously furnished to us by the Carbide and Carbon Chemicals Corporation, New York, N. Y.