[CONTRIBUTION FROM WESTERN REGIONAL RESEARCH LABORATORY[1]]

Esterification of Subtilin and its Effect on Solubility and *in vitro* Bacteriostatic Activity

BY J. F. CARSON, E. F. JANSEN AND J. C. LEWIS

This paper[2] describes the preparation of a series of methyl, ethyl, hydroxyethyl and hydroxypropyl esters of the polypeptide, subtilin. Subtilin is produced by a strain of *Bacillus subtilis* in submerged fermentation[3] and is bacteriostatically active *in vitro* and *in vivo* against many Gram-positive organisms[4] and *in vitro* against several acid-fast bacteria, including virulent strains of *Mycobacterium tuberculosis*.[5] Difficulties in administering the antibiotic are encountered because of its very low solubility in dilute salt solutions, as in animal tissues or in blood serum, and to its poor absorption from animal tissue.[6] In an attempt to overcome these disadvantages, the carboxyl groups of subtilin have been esterified with four different alcohols and the products with varying ester content have been examined with respect to bacteriostatic potency and solubility in physiological saline. A second purpose was to gain information regarding the essentiality of the different functional groups for antibiotic activity.

The procedure adopted for esterification was based on the method of Fraenkel-Conrat and Olcott[7] and consisted of dispersing the polypeptide in one hundred parts of anhydrous methanol, ethanol, ethylene glycol or propylene glycol, containing hydrogen chloride in concentrations of 0.02–0.06 *N*. The reactions were allowed to proceed for varying periods of time at temperatures 0 and 25° and the isolated derivatives were analyzed for ester content, amino nitrogen and amide nitrogen. Bacteriostatic potencies against *Micrococcus conglomeratus* (MY), *Streptococcus faecalis* (ATCC 7080), and *Staphylococcus aureus* (H) were determined, and the solubilities of the esters in 0.85% sodium chloride at *p*H 7.3–7.4 and 35° were measured. The extremely mild conditions employed in the reactions permitted close control of the esterification to the extent that a series of derivatives was obtained with a wide variation in ester content. It was also possible in reactions with methanolic hydrogen chloride to adjust conditions so that the reaction on the one hand would lead exclusively to esterification of carboxyl groups or the reaction of esterification could be accompanied by methanolysis of a portion of the amide groups without appreciable destruction of peptide linkages.

In Table I are recorded the conditions for esterification with methanol, ethanol, ethylene glycol and propylene glycol, relative bacteriostatic activities, and solubilities in physiological saline.

Experimental

Materials.—Subtilin was prepared as described elsewhere[3] from submerged cultures of *B. subtilis*. The polypeptide was de-ashed by passage of a 5% aqueous solution through a cation exchanger, IR-100[8] (hydrogen cycle), and recovered by lyophilization of the effluent. Electrophoretic analysis indicated that the subtilin was homogeneous.[3b] Attempts to obtain evidence of inhomogeneity by fractional dialysis against salt solutions have failed.[3b] On the other hand, investigations at the Research Laboratories of Merck & Co., Rahway, New Jersey, by counter-current distribution, show that our samples of subtilin are probably about 90% pure on the basis of the increased bacteriostatic potency of the principal fraction. The amino acid composition of subtilin is to be published elsewhere.

Results of analyses of the lyophilized, de-ashed subtilin, in terms of equivalents[9] per gram of dry subtilin × 10^4, were as follows: Kjeldahl nitrogen, 113; sulfur, 15.0; amino nitrogen (Van Slyke), 12.7; amide nitrogen, 15.7; carboxyl,[10] 6–7.

Methanol, Ethanol, Ethylene Glycol and Propylene Glycol-Hydrogen Chloride.—Methanol and ethanol were reagent-grade absolute alcohols. The methanol- and ethanol-hydrogen chloride solutions were prepared by bubbling dry hydrogen chloride into the alcohol. Ethylene glycol and propylene glycol, technical grades, were purified and dried by distillation with toluene. The acidified glycols were prepared by addition of a calculated quantity of concentrated reagent hydrochloric acid (10 *N*) to the glycols.

Esterification with Methanol.—All reactions were conducted with 1% solutions of subtilin in the reagent. The polypeptide, although insoluble in neutral methanol, dissolved in a few minutes in the acidified alcohol. The esters were isolated in the following manner: hydrochloric acid was removed by stirring the methanol solution with the acid-absorbing resin, IR-4B in the amino form, until the apparent *p*H, measured with a glass electrode, was 5–6. The solution, after removal of the resin, was concentrated to dryness *in vacuo* (Dry Ice-trap and bath temperature 25–30°). The residual ester was dissolved in water to make a 1–2% solution and again treated with ion exchangers to ensure complete removal of ammonium ion, resulting from possible amide splitting, which would interfere with amide and Van Slyke amino nitrogen determinations. The solution was stirred with about 5% of its weight of Amberlite IR-100 for six to eight hours. The solution was then lyophilized, unless the *p*H was less than 3.5, in

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(2) J. C. Lewis and E. F. Jansen, *Fed. Proc., Fed. Amer. Soc. Expt. Biol.*, **6**, 270 (1947).

(3) (a) J. J. Stubbs, *et al.*, *Arch. Biochem.*, **14**, 427 (1947); (b) H. L. Fevold, *et al.*, *ibid.*, **18**, 27 (1948).

(4) A. J. Salle and G. J. Jann, *Proc., Soc. Expt. Biol. Med.*, **60**, 60 (1945); **62**, 40 (1946); **63**, 41, 519 (1946); H. Eagle, *et al.*, *J. Bact.*, **55**, 347 (1948); D. M. Reynolds, unpublished data.

(5) A. J. Salle and G. J. Jann, *Proc. Soc. Expt. Biol. Med.*, **60**, 60 (1945); H. H. Anderson and S. C. Wong, *Tuberculol.*, **8**, 77 (1946); S. C. Wong, *et al.*, *J. Lab. Clin. Med.*, **32**, 837 (1947).

(6) R. H. Wilson, *et al.*, *Fed. Proc., Fed. Amer. Soc. Exptl. Biol.*, **7**, 266 (1948).

(7) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(8) Resinous Products and Chemical Co., Philadelphia, Pennsylvania.

(9) The term equivalents/g. × 10^4 as used in this paper refers to the dry solid isolated product whether subtilin or a subtilin derivative. This will be abbreviated to "equivalents."

(10) Titration between *p*H 6 and 2 by procedure of R. M. Harriott, M. L. Anson and J. H. Northrop, *J. Gen. Physiol.*, **30**, 208 (1946).

which case it was first adjusted to 5–6 by stirring for a few minutes with Amberlite IR-4B. The esters were all lyophilized twice to ensure complete removal of any adsorbed methanol. As an alternative, the esters could be precipitated from methanol solutions by the addition of three volumes of ethyl ether. The recovered esters were then dissolved in water, treated with ion exchangers, and lyophilized as before. Yields were generally 95% or better.

Esterification with Ethanol.—Subtilin did not dissolve appreciably in ethanolic hydrogen chloride at any stage of the reaction and hence vigorous agitation on a shaking machine was employed. To terminate the reaction, four volumes of acetone were added to the suspension and the ester isolated by filtration or centrifugation. Further treatment with ion exchangers and final lyophilization followed the general procedure used in methylation.

Esterification with Ethylene and Propylene Glycols.—Subtilin dissolved in the acidified ethylene glycol after it had been shaken a few minutes, while about twenty-four hours were required for solution in propylene glycol–hydrochloric acid. The hydroxyethyl and hydroxypropyl esters were isolated by pouring the clear reaction solutions into 8 or 9 volumes of technical acetone, followed by centrifugation or filtration. The esters were then washed 4 or 5 times with reagent acetone. It was found necessary to wash the esters thoroughly with acetone, since traces of glycol could not be removed completely by lyophilization and interfered seriously in analyses of the esters for glycol content. The esters were then dissolved in approximately 50 parts of water, the *p*H was adjusted to 5–6 with IR-4B, and the solution was then treated with IR-100 as before. Yields were almost quantitative.

TABLE I

THE ESTERIFICATION OF SUBTILIN

Ester	Reaction conditions: Concn., N	Temp., °C.	Reaction time, hours	Analytical (equiv./10^4 g.) Nitrogen, %: Ester content[a]	Amide[b]	Amino[c]	Relative bacteriostatic potency[d]: MY	*Strep.*	*Staph.*	Ratio of solubility to solubility of subtilin[e]
Subtilin				<0.1	15.7	12.7	(100)	(100)	(100)	(1.0)
Methyl	0.03 hydrogen chloride in methanol	0	5	1.5	15.9	12.8	180	160	47	0.8
			16	2.7	16.2		240	220	51	
			29	3.9	16.1		280	240	52	
			51	5.3		12.1	320	230	60	
			72	5.6		12.1	390	230	60	1.8
			98	6.0			350	250	59	1.8
			168	6.6	15.7	12.3	340	280	58	
			360	7.2		12.0	270	280	58	2.7
			473	7.3	15.0	12.3	250	290		
Methyl	0.03 hydrogen chloride in methanol	25	6	4.3			280	230	50	
			17	6.5	15.6	13.1	330	250	51	
			29	7.8	15.3	13.3	230	180	57	2.5
			44	8.6	14.9	13.5	210	230	52	4.1
			72	9.7	14.0	12.7	150	150	42	31.0
			98	11.0	13.8	12.6	120	120	30	46
			164	11.9	12.7		91	110	24	
			193	13.7	12.5	12.1	73	93	23	
Ethyl	0.06 hydrogen chloride in ethanol	0	40	1.0	15.4	12.9	80	84		
			112	1.3	15.6		80	87		
			162	1.3			94	90		
			265	3.6	15.4	13.0	85	110		
Ethyl	0.03 hydrogen chloride in ethanol	25	40			12.0	68	66	39	
			76	5.1	15.3	11.4	27	78	20	0.9
			120	5.7	15.3	11.4	41	64	26	
			195	6.3	15.2	10.3	30	58	16	1.1
			240	6.8	15.5	10.3	30	41	16	1.5
			317	7.3	15.2	10.2	15	29	11	1.6
Hydroxyethyl	0.06 hydrogen chloride in ethylene glycol	0	23				99	110	75	
			50	1.4		12.9	140	120	74	
			74		15.3		120	100	68	
			118	2.4			170	180	84	1.9
			169	2.6	15.0	13.0	200	200	96	
			213	3.0	15.0	13.1	230	200	88	2.5
			333	2.7	15.1	12.8	240	280	110	
Hydroxyethyl	0.02 hydrogen chloride in ethylene glycol	25	40	2.4	15.6	12.0	150	190	89	1.6
			69	2.8	15.8	11.7	190	200	89	2.0
			89	3.4	15.3	10.9	170	190	89	2.1
			113	4.0	15.3		130	150	67	2.9
			170	4.3	15.0	10.5	82	92	40	4.9
			220	4.9	15.8	10.3	71	94	36	34

TABLE I (*Continued*)

Ester	Reaction conditions: Concn., N	Temp., °C.	Reaction time, hours	Analytical (equiv./10^4 g.) Ester content[a]	Nitrogen, % Amide[b]	Amino[c]	Relative bacteriostatic potency[d] MY	*Strep.*	*Staph.*	Ratio of solubility to solubility of subtilin[e]
Hydroxy-ethyl	0.03 hydrogen chloride in ethylene glycol	25	48	3.2		11.3	130	140		
			70	4.0		10.7	130	170		3.3
			94	4.3		10.3	94	140		3.6
			141	4.6		10.1	54	71		34
			186	4.6		10.0	33			
Hydroxy-propyl	0.06 hydrogen chloride in propylene glycol	0	65	1.5	14.9	12.2	42	29	21	
		0	113		15.0	12.2	48	39	22	
		0	161	1.8	15.6	12.8	57	43	23	
			242	2.1	15.6	12.7	60	51	23	1.2
Hydroxy-propyl	0.03 hydrogen chloride in propylene glycol	25	38	4.0	15.1	11.4	47	38	25	1.4
			86	6.5	15.2	11.2	29	40	15	2.4
			114	6.7	15.4	11.1	20	34	13	2.2
			155	7.3	15.4	10.3	16	27	8	5.9
			206	7.3	15.3	10.4	12	23	8	6.1
			253	7.5	15.7	10.4	9	11	5	7.8

[a] Methoxyl and ethoxyl contents were determined by the Zeisel method as modified by Clark with a 30-minute reaction period (E. P. Clark, *J. Assoc. Offic. Agr. Chemists*, **15**, 136 (1932). Hydroxyethyl and hydroxypropyl ester contents were determined by alkaline hydrolysis, distillation of the resulting glycol as the toluene azeotrope and analyses of the glycol in the distillate by oxidation with periodic acid and titration of the excess periodate by the sodium arsenite–iodine procedure (E. L. Jackson, "Organic Reactions," Vol. II, 341–375 (1944)). It was found that the blank value with subtilin alone was less than 0.1 equiv./10^4 g. and on addition of measured quantities of ethylene or propylene glycol to subtilin or subtilin glycol esters, the glycol recovered was 95–97% of the calculated amount. Duplicate determinations were within 5%. [b] Amide nitrogen was determined by an adaptation of the findings of Rees (*Biochem. J.*, **40**, 632 (1946)). Samples were hydrolyzed by allowing one part of the polypeptide in 20 parts of reagent hydrochloric acid (10 *N*) to stand for 200–220 hours at 35°. Ammonia was then determined by neutralizing the solutions and distilling at *p*H 10 in a borate buffer. With subtilin the amide ammonia reached a constant value after 190–200 hours of hydrolysis. [c] Amino nitrogen was measured by the Van Slyke manometric procedure with 15-minute reaction period (D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929)). [d] *MY*, *Strep.*, and *Staph.* refer, respectively, to *Micrococcus conglomeratus* (MY), *Streptococcus faecalis* (A. T. C. C. 7080) and to *Staphylococcus aureus* (H). Microbiological assays were performed by the turbidimetric method of J. C. Lewis, *et al.*, *Arch. Biochem.*, **14**, 437 (1947). [e] The solubilities were measured in 0.85% sodium chloride solution at *p*H 7.3–7.4 and 35°. Solutions of the derivatives in 0.85% sodium chloride were adjusted to a *p*H of 7.3–7.4, allowed to stand eighteen hours at room temperature, and then equilibrated for two hours at 35° before centrifuging. Solubilities were calculated from Kjeldahl nitrogen determinations on aliquots of the saturated solutions. Since solubilities varied somewhat with the amount of solid phase present, quantities were used so as to give 10–20% excess of undissolved material at *p*H 7.3–7.4. Solubilities, as determined by nitrogen analyses, were checked frequently by bacteriostatic assay. The agreement was usually within 20%. The solubility of subtilin under these conditions was 0.09 to 0.1%.

Discussion

Esterification of Subtilin with Methanol.—At 0° in 0.03 *N* methanolic hydrogen chloride, a maximum methoxyl content of 7.3 equivalents was introduced with no appreciable loss in amide nitrogen or change in amino nitrogen. Since the methoxyl introduced agrees approximately with the carboxyl content (6–7 equivalents), of unesterified subtilin, the reaction under these conditions is specific for esterification of carboxyl groups. Bacteriostatic potencies to *M. conglomeratus* and to *Strep. faecalis* are increased even with slight esterification and reach maxima of about four- and threefold enhancements, respectively, at ester contents of 5–6 equivalents.[11] Activity toward *Staph. aureus* unexpectedly showed a substantial initial decrease when only 1.5 equivalents of methoxyl were introduced, followed by a slight increase with increasing ester content. Solubilities of the esters at maximum activity were increased about twofold over subtilin.

(11) Methyl esters with increased bacteriostatic activity could also be prepared by esterification in 80% methanolic hydrochloric acid 0.1–0.3 *N* but the reaction was more difficult to control and enhancement was usually less than in absolute methanol.

At 25° in 0.03 *N* methanol–hydrogen chloride, the introduction of methoxyl groups is not terminated at 7 equivalents when, presumably, esterification of the carboxyl groups is complete, but increases to the extent that after 193 hours, 13.7 equivalents of methoxyl are introduced. For esters with methoxyl contents of 8.6 equivalents or greater, amide nitrogen is decreased, thus indicating that the increased methoxyl contents are obtained mainly by methanolysis of amide linkages. The loss in amide is not quite stoichiometric with the increase in methoxyl. This reaction is in marked contrast to the reaction at 0°, where only carboxyl groups are attacked. Apparently the amide groups of subtilin are far more susceptible to cleavage by alcohols than are most protein amide groups.[7,12] Bacteriostatic potencies to *M. conglomeratus* and to *Strep. faecalis* attain maxima of three- and twofold enhancements, respectively,

(12) Although protein amide is generally associated mainly with the glutamic and aspartic acid residues, the source of amide nitrogen of subtilin is not known, since the diamino dicarboxylic sulfur acid, lanthionine, occurring in subtilin hydrolysates (G. Alderton, unpublished results) could exist partly as an amide. Actually, the amide nitrogen content of subtilin is greater than the combined glutamic and aspartic acid contents.

over subtilin at 6–7 equivalents of ester content, while activity to *Staph. aureus* shows the same initial decrease in the first stages of the reaction as was experienced with esterification at 0°. As the methoxyl content increases at the expense of amide groups, potencies to the three test organisms undergo a gradual decrease from the maxima; however, even at an ester content of 13.7 equivalents, activities to *M. conglomeratus* and to *Strep. faecalis* are only slightly less than the activity of subtilin. Free carboxyl groups and some of the amide groups are therefore not essential for bacteriostatic activity toward these two organisms. The effect of esterification and methanolysis on solubility is marked. The derivative with an ester content of 8.6 equivalents was soluble in physiological saline to about 0.4%, representing a fourfold increase over the solubility of subtilin, while the ester with 9.7 equivalents of methoxyl was soluble to the extent of 2.95% or approximately 30 times that of subtilin. The sudden increase in solubility appears to occur at a point somewhat beyond that for complete esterification where some amide loss has already occurred,[13] but amide and carboxyl determinations are not sufficiently precise to prove this point.

Esterification of Subtilin with Ethanol.—Esterification with ethanol–0.06 *N* hydrogen chloride at 0° was very slow, yielding a maximum of only 3.6 equivalents of ethoxyl after 265 hours. Activities toward *M. conglomeratus* and *Strep. faecalis* were slightly decreased. At 25° in ethanol–0.03 *N* hydrogen chloride, a period of 317 hours were required to introduce 7.3 equivalents of ethoxyl. Alcoholysis of amide groups did not take place, in contrast to the reaction with methanol at 25°. The amino nitrogen (Van Slyke) showed a progressive decrease from 12 equivalents after forty hours to 10.2 equivalents after 317 hours. The decrease in amino nitrogen[14] is surprising in view of the fact that under similar conditions in methanolic hydrogen chloride; the amino content was practically unchanged. If the decrease in amino nitrogen is the result of splitting of residues and loss through absorption on the ion exchangers used in purification, the fragments lost would necessarily be rich in amino nitrogen and small in size, since the yields of isolated products are almost quantitative. Bacteriostatic activities to the three test organisms were progressively decreased with increasing esterification, and the effect on solubility was slight. It is possible that the decrease in activity is associated with the decrease in amino content rather than with esterification of carboxyl groups.

Esterification with Ethylene Glycol.—Reaction of subtilin with ethylene glycol–0.06 *N* hydrochloric acid at 0° was very slow, extended reaction yielding esters with 3 equivalents of hydroxyethyl. No change in amide or amino nitrogen could be detected. Bacteriostatic activity to *M. conglomeratus* and to *Strep. faecalis* increased on esterification to maxima of two- to threefold enhancements over subtilin when the ester content reached 2.7 to 3.0 equivalents. Activity to *Staph. aureus* showed an initial decrease, followed by a gradual increase, as was experienced with the methyl and ethyl esters. Solubilities in physiological saline were increased slightly more than with methyl esters of the same ester content.

Esterification of subtilin in 0.02 *N* hydrochloric acid–ethylene glycol at 25° yielded a series of hydroxyethyl derivatives with ester contents varying from 2.4 to 4.9 equivalents as the reaction time varied from forty to 220 hours. Activities to *M. conglomeratus* and to *Strep. faecalis* showed maximum enhancements of twofold at hydroxyethyl contents of 2.8 to 3.4 equivalents and gradually decreased on continued reaction. There was no appreciable loss in amide groups, but the amino nitrogen showed a progressive decrease from 12.0 equivalents after forty hours to 10.3 equivalents after 220 hours in agreement with esterifications with ethanol but in contrast to esterification with methanol. It is possible that the decreased amino nitrogen is the result of interference of the hydroxyethyl groups in the Van Slyke nitrous acid procedure; however, esters prepared at 0° and containing 2.7–3.0 equivalents of hydroxyethyl showed no decrease in amino nitrogen.[15] The increase in solubility when the hydroxyethyl content changes from 4.3 to 4.9 equivalents is remarkable, and is in contrast with esterification with methanol, which requires an ester content of 9 equivalents or more for comparable solubility. The changes in solubility in this case cannot be explained on the basis of shifting of the isoelectric point. These results were confirmed by esterification in 0.03 *N* hydrochloric acid–ethylene glycol at 25°. The same general decrease in amino nitrogen was observed and the marked increase in solubility occurred at an ester content of 4.6 equivalents.

Esterification with Propylene Glycol.—The esterification of subtilin in propylene glycol–0.06 *N* hydrochloric acid at 0° was extremely slow with approximately 2 equivalents of hydroxypropyl introduced after 242 hours. There was no significant change in amide or amino nitrogen. Bacteriostatic potencies toward the three test organisms showed a large initial decrease, followed by a slight but significant increase in the case of *M. conglomeratus* and *Strep. faecalis*.

Esterification in propylene glycol–0.03 *N* hydrochloric acid at 25° yielded products with maximum ester contents slightly over 7 equivalents. There was no decrease in amide nitrogen, but the amino nitrogen content was decreased. Bacterio-

(13) The increased solubility of proteins on esterification is often ascribed to a shift in the isoelectric point, but it is doubtful that this explanation applies in the case of esterified subtilin.

(14) The consideration of footnote 12 may also apply to amino nitrogen.

(15) That unusually labile linkages exist in subtilin has also been demonstrated by treatment with very dilute alkali. Products are obtained with decreased amino nitrogen.

static potencies to all three test organisms were decreased. Solubility in physiological saline was gradually increased with increasing degree of esterification, but there was no sudden increase in solubility as was experienced in the hydroxyethyl esters.

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Summary

The esterification of subtilin with methanol is accompanied by an increase in bacteriostatic activity to *Micrococcus conglomeratus* and to *Streptococcus faecalis* and a decrease in activity to *Staphylococcus aureus*. The reaction when conducted at 0° is highly specific for esterification of carboxyl groups. At 25°, esterification of carboxyl groups is followed by methanolysis of some of the amide groups without splitting of peptide bonds. Greatly increased solubility is attained when the ester content is increased beyond that for complete reaction of the carboxyl groups.

Ethyl esters prepared under conditions comparable to methylation showed generally decreased bacteriostatic activity and no alcoholysis of amide groups. Some of the products show decreased amino nitrogen. Increase in solubility was only slight.

Hydroxyethyl esters of subtilin showed increased bacteriostatic activity toward *M. conglomeratus* and *Strep. faecalis*. Esterification of subtilin in ethylene glycol was not accompanied by loss of amide nitrogen, but some of the products showed a decrease in amino nitrogen. Although the carboxyl groups could not be completely esterified, some of the esters had greatly increased solubility in physiological saline.

Hydroxypropyl esters showed decreased bacteriostatic activity and only moderate increase in solubility. Esters prepared at 25° showed a decrease in amino nitrogen.

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2-Lepidyl Substituted Diamines

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In the course of an investigation on the preparation of N-substituted aminoalcohols, it was observed that replacement of a 2-pyridyl group by a 2-lepidyl substituent gave a compound having enhanced antihistaminic activity.[1] It seemed of interest, therefore, to determine what effect a similar replacement of the 2-pyridyl group in N,N - dimethyl - N′ - (2-pyridyl) - N′ - benzylethylenediamine,[2] and in other active 2-pyridyl substituted diamines[3-5] would have upon the histamine antagonistic activity.

The intermediate 2-lepidyl secondary amines were prepared by one of three general procedures. The method of choice, A, was used only in those cases where the N,N-substituted ethylene or propylenediamines were commercially available. Excellent yields of I and II were obtained by heating 2-chlorolepidine with excess alkylene diamine. Whitmore[6] and Huttrer[2] used the same type of reaction with 2-bromopyridine to prepare 2-amino-alkylaminopyridines. Their products were obtained in good yields when an excess of alkylene diamine was used. Their reactions, however, were run in pyridine as solvent, since poor yields were obtained in its absence, and in sealed tubes, since the reaction temperature was 140–160°. In this investigation it was found that the use of a considerable excess of alkylene diamine in the absence of any solvent eliminated the need for pressure vessels.

Methods B and C were used for the preparation of 2-benzylaminolepidine and analogous amines of structures III–XIII. Method B is an application of the procedure of Tschitschibabin[7a-c] as modified by Huttrer, *et al.*,[2] who replaced sodium amide with the more easily handled and commercially available lithium amide. Method C is an extension of the method of Tschitschibabin and Knunjanz[8] who prepared secondary amines by condensing 2-aminopyridine with benzaldehyde using formic acid as solvent and reducing agent. The method seems to be of special importance where the requisite substituted benzaldehydes are more readily available than the corresponding benzyl chlorides.

The tertiary amines were prepared by Methods

(1) Unpublished work of the author, to appear at a later date.

(2) Huttrer, Djerassi, Beears, Mayer and Scholz, THIS JOURNAL, **68**, 1999 (1946).

(3) N,N - Dimethyl - N′ - (*p* - methoxybenzyl) - N′ - (2 - pyridyl)-ethylenediamine; Bovet, Horclois and Walthert, *Compt. rend. soc. biol.*, **138**, 99 (1944); *C. A.*, **39**, 3070 (1945).

(4) N,N - Dimethyl - N′ - (2 - pyridyl) - N′ - (2 - thenyl) - ethylenediamine: (a) Weston, THIS JOURNAL, **69**, 980 (1947); (b) Ercoli, Schachter, Leonard and Solmssen, *Arch. Biochem.*, **13**, 487 (1947).

(5) N,N - Dimethyl - N′ - (2 - pyridyl) - N′ - (5 - halogeno - 2-thenyl)-ethylenediamine, where the halogens are bromine or chlorine; Clapp, Clark, Vaughan, English and Anderson, THIS JOURNAL, **69**, 1549 (1947).

(6) Whitmore, Mosher, Goldsmith and Rytina, *ibid.*, **67**, 393 (1945).

(7) (a) Tschitschibabin and Seide, *J. Russ. Phys.-Chem. Soc.*, **46**, 1216 (1914); *Chem. Zentr.*, **86**, I, 1064 (1915); (b) Tschitschibabin, Konowalowa and Konowalowa, *Ber.*, **54**, 814 (1921); (c) Tschitschibabin and Knunjanz, *ibid.*, **61**, 2215 (1928).

(8) Tschitschibabin and Knunjanz, *ibid.*, **64**, 2839 (1931).