The precipitate was dissolved in 100 ml. of acetone and water added drop by drop until the solution became cloudy. The flask was again kept in a refrigerator overnight. The precipitate was separated by centrifugation and washed with 95% alcohol, the liquid decanted and the precipitate washed with anhydrous ether. The precipitate was isolated by filtration and dried *in vacuo* over concentrated sulfuric acid. The decomposition point was observed at about 254°. No literature values on the decomposition point could be found.

Anal. Calcd. for $C_9H_{20}ON_7CrS_4$: N, 23.20. Found: N, 22.80, 23.50.

Ethanolamine Reineckate.—In one run, made under conditions identical to those employed by Barrenscheen (ref. 1), DL-methionine and ethanolamine were incubated with rat liver homogenate according to the protocol. See Flow Sheet labeled Protocol I. At the end of the incubation period the contents of the various flasks were combined and Barrenscheen's directions for the isolation of choline reineckate followed exactly. A precipitate similar in appearance to that of choline reineckate was obtained. After drying *in vacuo* at 100° this material melted with decomposition at about 170°.

Anal.[12] Calcd. for ethanolamine reineckate, $C_6H_{14}N_7OS_4Cr$: C, 18.95; H, 3.68; N, 25.79. Found: C, 18.91; H, 3.72; N, 25.04.

An authentic sample of ethanolamine reineckate prepared in this Laboratory melted with decomposition at about 170°.

PROTOCOL I

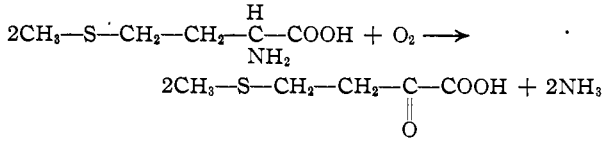
Source of enzyme: fresh rat liver homogenate in Krebs–Henseleit solution; substrate: DL-methionine in Krebs–Henseleit solution, ethanolamine neutralized with dilute HCl to *p*H 7.2; time of incubation one hour

Flask no.	Contents of each flask		Oxygen absorbed less endogenous oxygen: micromoles
2	DL-Methionine	12.8 mg.	1.8
3	Ethanolamine	40.0 mg.	1.6
4	10% Homogenate	1.0 ml.	1.5
5			1.9
6			1.7
7			1.8
		Average	1.7

(12) The microanalyses reported here were performed by Mrs. Mary H. Aldridge and Mr. Byron Baer.

Discussion

Examination of Table I reveals the following: In every experiment where a disappearance of methionine was observed, keto acid formation was also observed. The ratio of methionine disappearing to oxygen consumed in these cases was 2:1 (average of nine experiments). Assuming the reaction for the oxidative deamination of methionine to be



the methionine disappearing can be accounted for by oxidation to the keto analog. Supporting this view are the results with L-methionine (runs 12 and 13) which would not be affected by the D-amino acid oxidase.

The observation of Steensholt[5] that D-methionine is a better methyl donor in transmethylation, when judged by its disappearance as measured by the McCarthy–Sullivan method, must now be interpreted in a different light. The failure of rabbit liver homogenates, known to be low in D-amino acid oxidase, to cause any loss in methionine is in line with our other experimental evidence.

Examination of Table I shows that in no case was any choline produced, though from the amount of methionine disappearing sufficient choline could have been formed by transmethylation to be easily detected. In the only case in which a reineckate precipitate formed, the material was identified as the reineckate of ethanolamine.

COLLEGE PARK, MD. RECEIVED NOVEMBER 7, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Purification of Adrenocorticotropic Peptides by Carrier Displacement Chromatography

BY CHOH HAO LI, LUCIENNE ASH AND HAROLD PAPKOFF

The adrenocorticotropic peptides (ACTH peptides) prepared by peptic digest of sheep ACTH protein have been purified by fractional precipitation with trichloroacetic acid (TCA). It was found that the activity in the digest occurs in a 5–25% TCA precipitate. The ACTH activity in this precipitate can be abolished by further hydrolysis with pepsin and trypsin. Moreover, the 5–25% TCA precipitate can be further purified by the carrier displacement chromatographic technique. Using normal alcohols with 8 to 10 carbon atoms as the carriers, it was possible to obtain an active fraction having a specific activity of about 10–20 times that of the starting material. The amino acid composition of some of the purified fractions was investigated; one of these active fractions was found to have a low cystine content and to be essentially free from methionine and tyrosine.

We recently reported the analysis of a peptide mixture derived from the partial hydrolysis with pepsin of sheep adrenocorticotropic hormone (ACTH), by various chromatographic techniques.[1] It was found that a purified ACTH peptide fraction could be obtained by the carrier displacement procedure of Tiselius and Hagdahl.[2] However, although the fraction was chemically somewhat purified by this procedure, there was achieved no increase of biologic activity. In the present investigation, a plastic column was substituted for the stainless steel which had been used before, since it was thought that the previous difficulties had probably been caused by the reaction of the steel with HCl which in turn inactivated the hormone. When the plastic column was employed, not only was the ACTH activity totally recovered, but there also resulted an enhancement of activity: a fraction was obtained which had a specific activity of about 10–20 times that of the starting material.

Preparation and Properties of Preparation A2.—The 5% trichloroacetic acid (TCA) soluble fraction[3] of the partial

(1) C. H. Li, A. Tiselius, K. O. Pedersen, L. Hagdahl and H. Carstensen, *J. Biol. Chem.*, **190**, 317 (1951).

(2) A. Tiselius and L. Hagdahl, *Acta Chem. Scand.*, **4**, 394 (1950).

(3) Designated as Preparation S.

pepsin hydrolysate of the protein hormone[4] may be separated further into active and inactive fractions by increasing the TCA concentration to 25%. It was noted that the 5–25% TCA insoluble material contained practically all the ACTH activity, whereas the 25% TCA soluble fraction was low in biologic potency. An increase beyond 25% of the TCA concentration resulted in a decrease of the amount of precipitates formed, and at a 60% TCA concentration, no precipitation whatsoever occurred. The final product[5] of this simple fractionation possessed an activity greater than that of the starting material. The bioassay results are summarized in Table I.

TABLE I

BIOASSAY OF VARIOUS ACTH FRACTIONS OBTAINED FROM TCA[a] FRACTIONATION OF THE PEPTIC DIGEST OF THE PROTEIN HORMONE

Preparation	Fraction	Dose, μg.	Assay: No. of rats	Assay: Ascorbic acid depletion, mg./100 g. adrenals	ACTH equivalent,[c] μg.
ACTH	Protein	5	19	−121.9 ± 17.5[b]	5.4
S	5% TCA soluble of peptic digest	7	31	−138.9 ± 28.4	10.0
A	5–25% TCA ppt.	6	36	−150.8 ± 24.0	14.5
S2	25% TCA soluble	7	17	− 10.0 ± 21.7	<0.2
A2	Preparation A after ether extraction and lyophilization	4	16	−143.5 ± 19.0	10.5

[a] TCA, trichloroacetic acid. [b] Mean ± standard deviation. [c] The values are in terms of USP standard.

In order to remove the excess TCA in the active 5–25% TCA precipitate, the acidified solution was extracted repeatedly with peroxide-free ether or else put through an Amberlite IR-4B anion exchange column.[6] The former procedure gave rise to an HCl and TCA salt,[7] while a free ACTH base was obtained from the exchange resins.

The paper chromatogram of Preparation A2, on Whatman No. 4 filter paper according to the procedure of Consden, *et al.*,[8] using butanol–acetic acid (10%) as the developing solvent showed a decrease in concentrations of front ninhydrin-reacting spots as compared with those chromatograms obtained with the 5% TCA soluble fraction (Preparation S). Earlier studies[1] indicated that it is the immobile spot on the paper chromatogram which possesses most of the ACTH potency. It is therefore not surprising to find that Preparation A2 gives rise to a ninhydrin-reacting area occurring almost exclusively in the origin of the paper chromatogram.

The retention of biologic activity after partial hydrolysis of ACTH protein,[4,9] has been demonstrated previously. As may be seen in Table II a 1% solution of Preparation A2 in 6.0 *M* HCl at 37° for 6 hours does not destroy the hormonal activity; hydrolysis for a longer time, however, does decrease the potency. At 100°, the adrenal-stimulating activity of the hormone remains unchanged after incubation with 0.2 *M* HCl for 60 minutes. A decrease of ACTH potency occurs if the hormone solution in 1.0 *M* HCl is put into a boiling water-bath for one hour. From the paper chromatogram shown in Fig. 1, it is evident that hydrolysis did occur under these conditions.

TABLE II

EFFECT OF HCl–HEAT TREATMENT ON PURIFIED ACTH PEPTIDE PREPARATION A2

Conditions[a]	Assay at 2 μg. level: Ascorbic acid depletion, mg./100 g. adrenal	Estd. ACTH equiv.,[b] μg.
Untreated	−103, −118, −114, −102, −108	3.5
0.2 M, 60 min., 100°	−101, −127, −118, −125, −102, −151, −123, −115, −155	6.0
1.0 M, 30 min., 100°	−106, −98, −78, −68	1.7
6.0 M, 6 hr., 37°	−130, −180, −174, −93, −130, −92, −110, −132, −138	7.8
6.0 M, 8 hr., 37°	−42, −16, −97	0.5
6.0 M, 12 hr., 37°	−54, −48, −41, −55, −23	0.4
6.0 M, 19 hr., 37°	−24, −48, −23	0.3
6.0 M, 24 hr., 37°	+16, +63	0

[a] 10 mg. of ACTH peptide was dissolved in 1 cc. of 0.2 *M* HCl, or 1.0 *M* HCl, or 6.0 *M* HCl and kept at 37° for different periods. [b] The values are in terms of USP standard.

Reaction with trypsin abolishes the ACTH activity of Preparation A2. Incubation with pepsin for 2 hours does not destroy the potency of the hormone, but 4-hour digestion causes a decrease of the ascorbic-acid depleting activity of the hormone. The data are summarized in Table III.

TABLE III

EFFECT OF PEPSIN AND TRYPSIN ON PURIFIED ACTH PEPTIDE PREPARATION A2

Conditions	Ascorbic acid depletion, mg./100 g. adrenal	Equivalent ACTH equivalent,[c] μg.
Control	−103, −118, −114, −102, −108	3.5
Pepsin,[a] 2 hr.	−121, −118	5.0
Pepsin, 4 hr.	−60, −27, −67, −41	0.6
Trypsin,[b] 4 hr.	−4, −12, −27	<0.2

[a] 10 mg. of ACTH peptide and 0.5 mg. of crystalline pepsin were dissolved in one cc. of 0.01 *M* HCl and kept at 37° for 2, 4 or 24 hours. [b] 10 mg. of ACTH peptide and 0.5 mg. of trypsin were dissolved in 1 cc. of *p*H 7.0 phosphate buffer and kept at 37° for 4 hours. [c] The values are in terms of USP standard.

The amino acid content of a sample of Preparation A2 was estimated by microbiological technique.[10] Results given in Table IV show that practically all the known amino acids are present in the peptide preparation. When compared with the data obtained with the protein, the change in cystine content is striking: it decreases[11] from 8.6 to 0.9%. The amounts of methionine, aspartic acid, phenylalanine, proline and valine remain unchanged. A decrease of glutamic acid, glycine, leucine and threonine is evident, whereas there appears to be an increase in Preparation A2 of histidine and lysine.

There are good agreements between the values obtained by microbiological assay and those obtained by starch chromatography.[12] This may suggest the absence of D-amino acids in ACTH preparations.

Carrier Displacement Chromatography of Preparation A2.—Previous experiments[1] showed that the carrier displacement chromatographic technique of Tiselius and Hagdahl[2] is most readily applicable to the purification of the ACTH peptide mixture using normal alcohols with 8 to 10 carbon

(4) (a) C. H. Li, Conf. on Metabolic Aspects of Convalescence, Josiah Macy, Jr., Foundation, New York, 17th Meeting, 114 (1948); (b) C. H. Li and K. O. Pedersen, *Ark. Kemi.*, **1**, 333 (1950).

(5) Designated as Preparation A2.

(6) The resin was first washed with 3% Na_2CO_3 and then with distilled water. The TCA-containing peptide solution (300 mg. in 50 cc.) was then passed through the resin column (diameter, 1.1 cm.; length, 9 cm.) at a rate of 15 cc. per hour; the *p*H of the eluent was found to be approximately 8. No loss of N or of biologic activity was observed. The acid-free eluent was then lyophilized; the dry solid had 16.7% N and 0.2% chloride, indicating that the product was substantially free from trichloroacetic acid.

(7) The HCl salt of the ACTH peptides may be further fractionated by methanol–ether solvents. The HCl salts were found to be soluble in absolute methanol. A precipitate, formed when an equal volume of ether was added to the methanol solution, contained most of the ACTH activity.

(8) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **38**, 224 (1944).

(9) C. H. Li, THIS JOURNAL, **73**, 4146 (1951).

(10) The analysis was carried out in the Shankman Laboratories, Los Angeles.

(11) J. I. Harris and C. H. Li (*Federation Proc.*, **10**, 195 (1951)) have already demonstrated that partial desulfurization of ACTH preparations did not cause loss of hormonal activity.

(12) R. Mendenhall, Ph.D. Thesis, University of California, Berkeley, 1951.

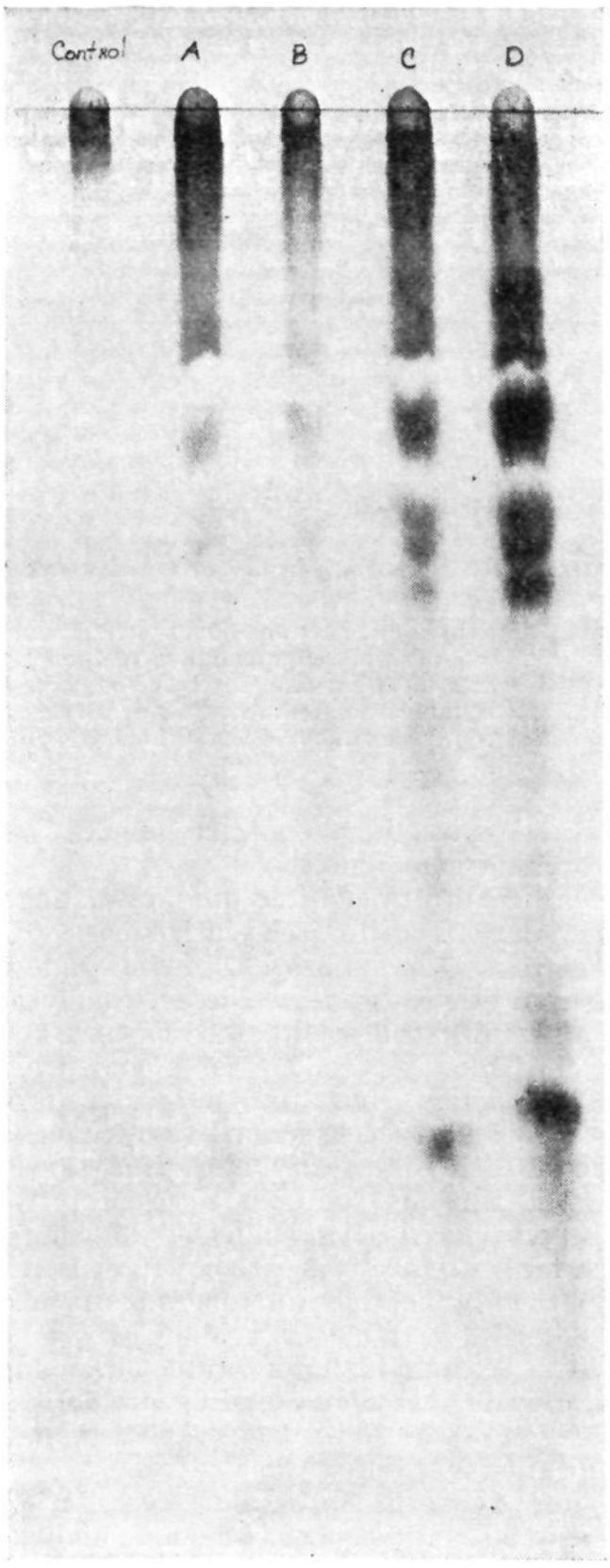


Fig. 1.—Paper chromatography of the HCl hydrolysate of Preparation A2: A, 1% solution in 6 *M* HCl, at 37° for 6 hours; B, 1% solution in 0.2 *M* HCl at 100° for 1 hour; C, 1% solution in 1 *M* HCl at 100° for ½ hour; and D, 1% solution in 1 *M* HCl at 100° for 1 hour.

atoms as the carriers. By modifications of the original procedure, it has become possible to recover quantitatively the biologic activity and to obtain fractions 10–20 times more active than the starting material.

The chromatographic column was made of plastic (Lucite) sections instead of the stainless steel previously employed. The adsorbent was a mixture of 1 part of Darco-G-60 and 9 parts of infusorial earth. Both the charcoal and the infusorial earth were washed thoroughly before use, first with HCl and then with water. In a typical experiment, 200 mg. of Preparation A2 was dissolved in 5 cc. of 1% *n*-octyl alcohol and introduced into the column[13] by suction. It was followed by 7 cc. of 2% *n*-nonyl alcohol, with a volume of

(13) The column was washed with 100 cc. of 1% *n*-octanol (in 50% ethanol containing 0.1 *N* HCl) before the material was introduced.

TABLE IV

AMINO ACID CONTENT OF ACTH PREPARATIONS

Values in g. per 100 g. protein or peptides

Amino acids	Protein		Peptides	
			Preparation A2	Preparation C
	Micro-biological assay[a]	Starch chromatography[b]	Micro-biological assay	Starch chromatography
Alanine	..	7.0	..	3.3
Arginine	8.7	10.3	6.9	11.6
Aspartic acid	6.7	6.8	6.4	9.9
Cystine	8.6	8.2	0.9	1.0
Glutamic acid	15.6	15.0	10.5	15.7
Glycine	8.0	8.7	5.0	6.1
Histidine	1.3	1.3	2.9	7.0
Isoleucine	3.1	3.0	2.3	1.9
Leucine	7.8	7.4	4.4	4.9
Lysine	5.0	5.3	9.8	16.8
Methionine	1.9	0.9	2.0	Nil
Phenylalanine	4.0	4.1	3.9	5.5
Proline	8.2	9.6	8.2	6.9
Serine	6.0	6.7	4.3	5.4
Threonine	3.2	3.6	1.9	3.8
Tyrosine	2.4	3.0	3.2	Nil
Valine	3.4	3.5	2.8	6.4

[a] Taken from ref. 9. [b] Taken from Mendenhall.[12]

70 cc. of 2% *n*-decyl alcohol for displacement. All higher alcohols were dissolved in 50% ethanol containing 0.1 *N* HCl. The column was constructed of 11 sections of 2 × 6280, 2 × 3140, and 7 × 1570 cu. mm. The displacer was delivered by a pressure of about 40 lb. per sq. in., at a rate of approximately 5 cc. per 30 minutes. The fractions were analyzed individually for their nitrogen content and biologic potency. It may be seen in Fig. 2 that nearly 70% of the nitrogen appeared in the first 40 cc. of the effluent and had very little adrenal ascorbic acid depleting activity. A maximal ACTH activity occurred between the fractions of 50–55 cc. having an average ACTH equivalent of 12 μg. per 0.1 μg. nitrogen. It should be noted that both nitrogen content and biologic activity were recovered in this experiment.

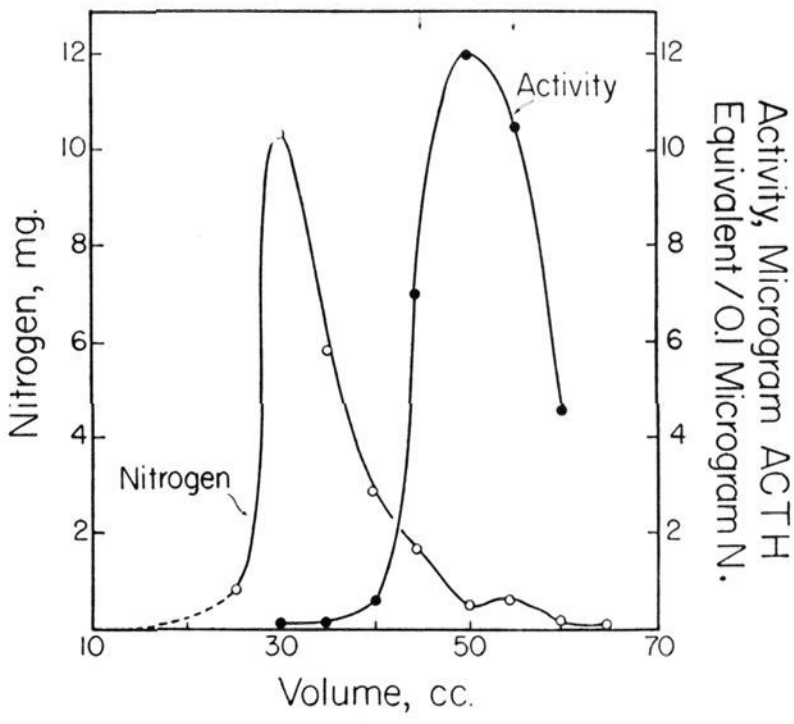


Fig. 2.—Nitrogen and ACTH activity distribution in various fractions obtained from the carrier displacement chromatography of Preparation A2.

A sample of the highest active fraction (denoted as Preparation C) obtained by the carrier displacement technique was subjected to ultracentrifugal analysis.[14] It was found

(14) The authors are indebted to Dr. K. O. Pedersen of Uppsala for the ultracentrifugal analysis.

that the material did not behave as a homogeneous substance and that the average molecular weight as computed by the Archibald[15] procedure was approximately 2000.

The amino acid content of Preparation C was investigated by the starch column technique of Moore and Stein[16]; the results obtained are summarized, together with other data, in Table IV. When it is compared with Preparation A2 in regard to amino acid content, it may be noted that Preparation C is considerably higher in histidine, lysine, arginine, valine and threonine. The preparation is essentially free from methionine and tyrosine.

(15) W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204 (1947).
(16) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

Acknowledgment.—The authors wish to express their thanks to Professor Arne Tiselius of Uppsala for many helpful discussions. We are also indebted to the U. S. Public Health Service, the Lasker Foundation, the Armour Laboratories, Merck and Company, Inc., and the Eli Lilly Company for research grants.

BERKELEY, CALIFORNIA RECEIVED NOVEMBER 2, 1951

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

Terramycin. II. Alkaline Degradation

BY R. PASTERNACK, A. BAVLEY, R. L. WAGNER, F. A. HOCHSTEIN, P. P. REGNA AND K. J. BRUNINGS

The alkaline degradation of the antibiotic terramycin produces ammonia, dimethylamine, carbon dioxide, acetic acid and a number of nitrogen-free compounds. Utilizing differences in acid strength, the following compounds have been isolated from the nitrogen-free fractions: (1) a strongly acidic compound $C_{13}H_{12}O_6$ which has been named terracinoic aicd, (2) 7-hydroxy-3-methylphthalide, (3) a phenolic compound, $C_{12}H_{10}O_3$ and (4) an as yet impure crystalline fraction with the acidity of an aliphatic carboxylic acid. Fractionation of the products of the caustic fusion of terramycin has yielded salicylic acid, *m*-hydroxybenzoic acid and succinic acid.

Terramycin,[1,2] the broad spectrum antibiotic isolated from culture broths of *Streptomyces rimosus*, is readily degraded by the action of alkali and of acid.[3] Alkaline treatment appears to result in the more drastic cleavage of the molecule and to yield products which are either identifiable as such or lend themselves well to structural determination. This paper describes in detail methods of carrying out aqueous alkaline degradations of terramycin and the identification of the products isolated thus far.

On boiling a 20% aqueous sodium hydroxide solution of terramycin, one mole each of ammonia and dimethylamine are evolved within 24 hours. When the hydrolysis is carried out in sodium carbonate, the evolution of the volatile bases is slow and incomplete. In acid hydrolysis, which will be the subject of a subsequent paper, dimethylamine appears to be more readily liberated than ammonia. Dimethylamine can also be cleaved selectively from the molecule by reduction in zinc and glacial acetic acid.[3]

Acetic acid (0.4 mole) and carbon dioxide (0.7 mole) have also been isolated from the degradation of terramycin in 20% aqueous sodium hydroxide.

When the alkaline hydrolysis is carried out in an inert atmosphere in the presence of zinc, a number of nitrogen-free products can be isolated after completion of the evolution of ammonia and dimethylamine. The method of separation takes advantage of the differences in the acid strengths of these degradation products and involves preliminary extraction of the acidified reaction mixture with ether, followed by successive extraction of this ether solution with *p*H 5.5 buffer and aqueous sodium bicarbonate. The ether solution remaining after these extractions contains several weakly acidic phenolic compounds.

The principal nitrogen-free product of the alkaline degradation can be isolated from the *p*H 5.5 buffer extract. Crystallization of the crude compound from ethyl acetate yields a strongly acidic white crystalline compound, melting at 233–234° with evolution of gas. This compound, which is optically inactive, has the molecular formula $C_{13}H_{12}O_6$ and has been named terracinoic acid. Terracinoic acid loses carbon dioxide slowly on heating in aqueous solution, a property which must be considered in crystallizing the compound from water. Complete decarboxylation is readily accomplished by heating in strong mineral acids to yield decarboxyterracinoic acid, $C_{12}H_{12}O_4$, m.p. 169–170°. A methyl ester of terracinoic acid forms very readily in methanolic hydrogen chloride and esterification occurs to a considerable extent when crystallization is attempted in alcohols.

The use of zinc in the alkaline degradation ensures a relatively smooth reaction and better yields but is not essential for the formation of terracinoic acid. Yields are not markedly affected by decreasing concentration of alkali and a yield of 30% has been obtained from a reaction run in 4% aqueous sodium hydroxide. However, no terracinoic acid has been isolated when the degradation is carried out in alcoholic sodium hydroxide.

From the ether solution remaining after the removal of terracinoic acid, a less acidic fraction can be extracted with aqueous sodium bicarbonate. No pure compound has been isolated from the bicarbonate extract which contains about 10% of the total weight of the alkaline degradation products. However, titration data on an impure crystalline product indicated pK_a values of about 5.5 and 9.3. Removal of this fraction from the ether extract is essential to the successful recovery of subsequent fractions.

When the ether solution remaining after the *p*H 5.5 buffer and sodium bicarbonate extractions is

(1) A. C. Finlay, G. L. Hobby, S. Y. P'an, P. P. Regna, J. B. Routien, D. B. Seeley, G. M. Shull, B. A. Sobin, I. A. Solomons, J. W. Vinson and J. H. Kane, *Science*, **111**, 85 (1950).
(2) P. P. Regna, I. A. Solomons, K. Murai, A. E. Timreck, K. J. Brunings and W. A. Lazier, THIS JOURNAL, **73**, 4211 (1951).
(3) R. Pasternack, P. P. Regna, R. L. Wagner, A. Bavley, F. A. Hochstein, P. N. Gordon and K. J. Brunings, *ibid.*, **73**, 2400 (1951).