about 10 drops per minute. The DNP-galactosaminitol had an $R = 0.4$ and DNP-glucosaminitol an $R = 0.1$.

Estimation of the Amino Sugars.—The bands of DNP-galactosaminitol and DNP-glucosaminitol were collected separately in flasks and evaporated to dryness *in vacuo*. The residues were dissolved in 10-ml. portions of water and suitable dilutions were made from this for reading in the Beckman spectrophotometer (model DU) at 3600 Å. The amounts of DNP-aminohexitols corresponding to these readings were then read off from a calibration curve made from known dilutions of reference material.

The absorptions of the DNP-aminohexitols were found to be proportional to concentrations within the range 2–12 μg./ml., with molar extinctions (ϵ) of 18.2×10^3 for DNP-glucosaminitol and 16.5×10^3 for DNP-galactosaminitol at 3600 Å. which corresponded to the absorption maximum.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Polymyxin B_1.[1] Fractionation, Molecular Weight Determination, Amino Acid and Fatty Acid Composition

BY WERNER HAUSMANN AND LYMAN C. CRAIG

RECEIVED MARCH 30, 1954

A sample of polymyxin B was hydrolyzed in order to recover the isopelargonic acid. However, countercurrent distribution showed two acids to be present, the expected 6-methyloctan-1-oic acid and an isoöctanoic acid. Purity studies by countercurrent distribution of the polymyxin B sample then gave two different polymyxin peptides, B_1 and B_2. On hydrolysis both gave α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. B_1 gave only the isopelargonic acid. B_1 and B_2 thus differ by the nature of the acid component. The molecular weight of B_1 was determined by the method of partial substitution and found to be 1150 ± 10%. Quantitative isolation studies by countercurrent distribution of a hydrolysate of B_1 showed it to contain 6 moles α,γ-diaminobutyric acid (the optical rotation indicated one mole of D and five moles of L), two moles L-threonine, one mole D-phenylalanine, one mole L-leucine and one mole of isopelargonic acid.

Introduction

During the course of structural studies with the polypeptide polypeptin[2] it was found that hydrolysis with sulfuric acid gave an ether extractable acid with a molecular weight approximating 150. Because of the general similarity of polypeptin to the class of polypeptide antibiotics known as polymyxins, the question of the relationship of this acid to the 6-methyloctan-1-oic acid[3] derived from the polymyxins was raised. This prompted a comparative study with one of the polymyxins. A generous sample of polypeptide material belonging to the polymyxin B type was made available to us through the kindness of Dr. P. Regna of Pfizer and Co. In 1949 Regna and co-workers[4] succeeded in purifying crude polymyxin B by chromatography on cotton sodium succinate followed by precipitation as the salt of the dye, polar yellow and final crystallization as the salt of naphthalene-β-sulfonate. The final product and the sulfate prepared from it behaved as a single pure substance when studied by paper chromatography. Acid hydrolysis followed by paper chromatography gave spots corresponding to α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. An ether extract of the hydrolysate gave the optically active 6-methyloctan-1-oic acid mentioned above.

Molecular weight studies[5] on polymyxin B purified by another method gave a value of 1280 ± 70. This figure is based on pressure–area studies with monomolecular films of the peptide.

When the sample of polymyxin B at our disposal was hydrolyzed and extracted with ether, an acid fraction was obtained as expected. However, when it was studied by countercurrent distribution two different acids, one of which was the expected 6-methyloctan-1-oic acid, were found to be present. This finding raised the question of either the purity of the sample of polymyxin B or of the accepted composition. It prompted a further study into the purity of the sample, its molecular weight and amino acid composition.

Experimental

Hydrolysis of an Unfractionated Sample of Polymyxin B.—When a sample of peptide material was hydrolyzed for 24 hours at 108° with 6 *N* hydrochloric acid the yield of fatty acid was poor. However, two-dimensional paper chromatography of the residue remaining after evaporation of the HCl, in the systems 2-butanol–ammonia and 2-butanol–formic acid[6] showed spots corresponding to α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. No other spots appeared. This result showed that the sample probably was not grossly contaminated with polymyxins of the other types.

For isolation of the fatty acid fragment 10 g. of the sample was hydrolyzed for 24 hours in 100 ml. of boiling 18% sulfuric acid under an atmosphere of nitrogen. Sulfuric acid was found to give a better yield than hydrochloric acid. The dark colored oil which had separated on the surface was extracted with ethyl ether. The ether extract was dried by freezing out the water in an acetone–solid carbon dioxide bath. The ether was removed by distillation through a Widmer column. A residue of 1.1 g. of a yellow oil with a rancid odor remained.

The residue was fractionated by countercurrent distribution in an automatic all-glass apparatus[7] using the system *n*-heptane–20% aqueous pyridine as shown in Fig. 1. For analysis 1-ml. aliquots of lower phase were directly titrated against phenolphthalein with 0.01 *N* sodium hydroxide. A distribution curve at 197 transfers is shown by the upper pattern of Fig. 1. After removal of the solute in the slowest moving band which proved to be sulfuric acid, the distribution was continued to 301 transfers. Analysis at this point gave the lower pattern of Fig. 1. Cuts 1a and 1b were removed at this stage and peak 2 was recycled for 200 more transfers. The material was then isolated in two cuts 2a and 2b from shoulder and peak, respectively. This pattern is not shown.

(1) Presented in part at the A.C.S. Meeting in Miniature, Metropolitan Long Island Subsection, February 20, 1953.

(2) W. Hausmann and L. C. Craig, *J. Biol. Chem.*, **198**, 405 (1952).

(3) S. Wilkinson, *Nature*, **164**, 622 (1949).

(4) P. P. Regna, I. A. Solomons, B. K. Forscher and A. E. Timreck, *J. Clin. Invest.*, **28**, 1022 (1949).

(5) A. V. Few and J. H. Schulman, *Biochem. J.*, **54**, 171 (1953).

(6) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).

(7) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

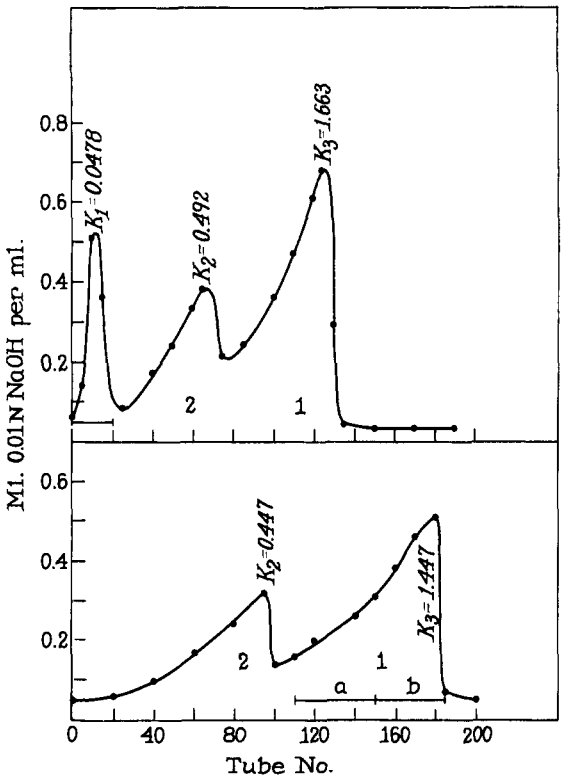


Fig. 1.—Distribution curves of fatty acids from polymyxin B: system, *n*-heptane–20% aqueous pyridine; charge, 1.135 g. in 4 tubes; transfers, 197 (top curve), 301 (bottom curve); ●—●, lower phase.

The acid solutes were converted to the sodium salts by neutralization with 0.1 *N* sodium hydroxide and recovered by evaporation of the solvent under reduced pressure.

Crystalline silver salts were prepared as follows. A 1 *N* aqueous silver nitrate solution was added to a concentrated solution of the respective sodium salt in water until no more precipitate formed. The white silver salt was filtered off, washed thoroughly with cold water and recrystallized in the dark from hot water. The crystalline amides were prepared by way of the acid chlorides. The latter were formed by boiling the sodium salts for 30 minutes under reflux with an excess of thionyl chloride. The excess reagent was removed by distillation. Isopropyl ether was added to the residue and the precipitate of sodium chloride was removed by filtration. A stream of ammonia was passed through the clear filtrate for 15 minutes, after which precipitation of the amide was complete. The derivative was filtered off and recrystallized from hot water.

The amides from both cuts of the most rapidly moving peak crystallized in colorless plates melting at 92–93° and were found to be identical. Similarly the amides from both cuts of the other band were indistinguishable. They crystallized in colorless plates at 110–111°. Analytical data for the amides and silver salts are given in Table I.

The amide of normal caprylic acid was prepared for comparison with the amide of the C_8 acid. A mixed melting point showed a depression. When both substances were studied by infrared absorption, differences were found in the region where C–H bending would be expected. An isooctanoic acid is thus indicated. No report of the occurrence of a C_8 acid in the polymyxin peptides has thus far been made as far as we are aware.

All data obtained for the most rapidly moving band including optical rotation and infrared measurements were in perfect agreement with those reported for the 6-methyloctan-1-oic acid known to be present in the polymyxins.

Countercurrent Distribution of Polymyxin B Sulfate.—A sample of 3 g. was distributed in the system 2-butanol–0.1 *N* HCl. After 1789 transfers, using the recycling procedure, the pattern in Fig. 2 was obtained. Analysis was

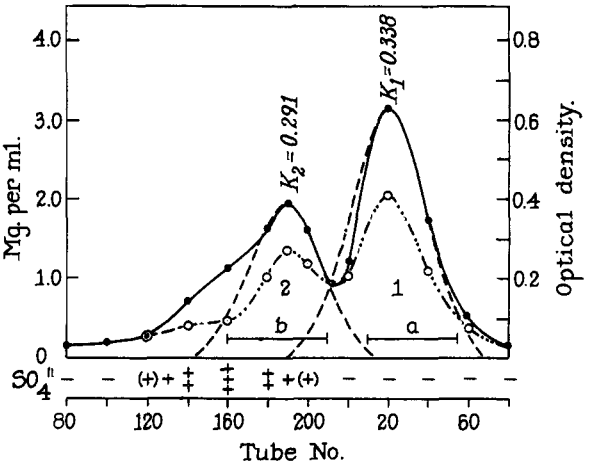


Fig. 2.—Distribution curves of polymyxin B: system 2-butanol–0.1 *N* HCl; phases, 10 ml. upper, 10 ml. lower; charge, 3 g. in tubes 1–15; transfers, 1789; ●—●, lower phase; O—O, optical density at 259 mμ of lower phase; ---, theoretical curve.

carried out by weight and absorption at 259 mμ, where phenylalanine has a maximum. The main peak was somewhat skewed probably due to a concentration effect. Cut a gave 1.5 g. of white fluffy hydrochloride upon rapid evaporation to dryness at 20° under high vacuum, redissolving in water and immediate lyophilization. Analytical data on this material, which we now propose to name polymyxin B_1, are given in Table I. The solute in peak 2, now called polymyxin B_2, showed signs of inhomogeneity due to an underlying sulfuric acid band. This H_2SO_4 came from the original peptide sulfate and was gradually stripped off and replaced by HCl during the distribution. The result of a qualitative test of the lower phases for sulfate ion with $BaCl_2$ is shown underneath the distribution curve. The band extends from tube 120 to 200, its peak occurring in tube 160. Polymyxin B_2 was isolated from cut b by prior precipitation of the sulfate with the theoretical amount of aqueous $BaCl_2$ solution. Upon centrifuging, the supernatant was evaporated to dryness at 20° under high vacuum and then lyophilized after dissolving the residue in water: 0.85 g. of a white, fluffy hydrochloride was obtained. Both peptides, B_1 and B_2, behaved like single, strongly basic compounds when studied by two-dimensional paper chromatography.[6] Hydrolysis and paper chromatography of samples of both compounds showed the presence of the same four amino acids previously mentioned. From the pattern of Fig. 2 it can be calculated that the original sample contained approximately 65% of B_1 and 35% of B_2.

Hydrolysis of Polymyxin B_1 in Sulfuric Acid.—One gram of material from cut a of band 1, Fig. 2, was hydrolyzed in 18% H_2SO_4 as before. The crude ether extract of the hydrolysate weighed 170 mg. In *n*-heptane it had an optical rotation $[\alpha]^{25}D$ +6.05° (*c* 4.6). The acidic material was subsequently distributed in the *n*-heptane–20% aqueous pyridine system. After 100 transfers the pattern in Fig. 3 was obtained. Beside the slow-moving sulfuric acid band, only one major component was present which travelled with a K = 0.66. Since this value, because of concentration effects, did not agree with the rate of travel of either acid 1 or 2 in Fig. 1, the material from the central band of Fig. 3 was isolated and its amide prepared for final identification. It crystallized from hot water in colorless plates, melting at 91–92°. When mixed with the amide of 6-methyloctan-1-oic acid, the melting point remained unchanged while in a similar experiment with the amide of the octanoic acid a depression was obtained.

Hydrolysis of Polymyxin B_1 in Hydrochloric Acid.—One gram of the peptide hydrochloride from cut 1a of Fig. 2 was hydrolyzed by refluxing for 24 hours in 100 ml. of 6 *N* HCl in a nitrogen atmosphere. The hydrolysate was exhaustively extracted in a Kutscher–Steudel apparatus with ether in order to remove the acid. The aqueous phase was evaporated to dryness *in vacuo*. The residue was distributed in the system *n*-butanol, 2-butanol, 5% HCl (1:1:2).

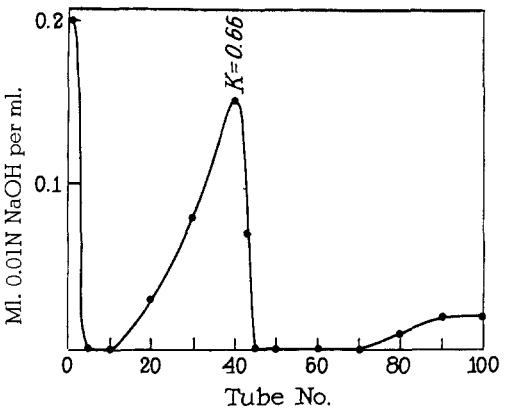


Fig. 3.—Distribution curve of fatty acid from polymyxin B_1: system, *n*-heptane–20% aqueous pyridine; phases, 10 ml. upper, 10 ml. lower; charge, 170 mg. in tube 1; transfers, 100; ●—●, lower phase.

Analysis after 211 transfers gave the curve shown in Fig. 4A. The band in tubes 80–130 which contained leucine and phenylalanine was also analyzed spectrophotometrically at a wave length of 259 mμ, where phenylalanine is known to have a maximum. The result shown in pattern B of Fig. 4 indicated that the peaks of the absorption and weight curves occurred practically together. This band was removed from the apparatus at this point and the slow moving peak containing α,γ-diaminobutyric acid and threonine was recycled until 726 transfers had been accomplished. Pattern C in Fig. 4 shows complete separation of the two amino acids. The two materials were isolated as the hydrochlorides by evaporation to dryness under reduced pressure.

The band containing leucine and phenylalanine hydrochloride was evaporated to dryness under reduced pressure, dissolved in H_2O and an excess of NH_4OH was added. After evaporation to dryness, the residue was distributed in the system *n*-butanol–H_2O. Pattern D in Fig. 4 shows almost complete separation of the two amino acids after 657 transfers. They were isolated in the usual manner and their optical rotations taken prior to crystallization. All four amino acids were crystallized and obtained in analytically pure form. All the analytical data are given in Table I.

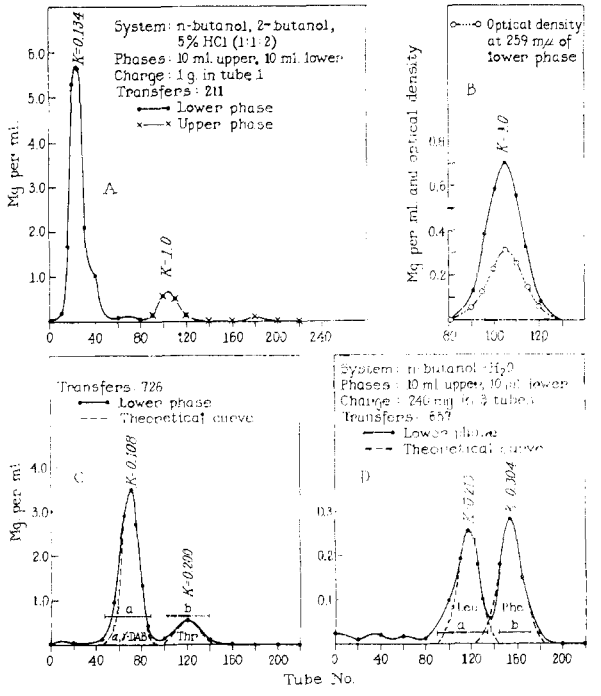


Fig. 4.—Distribution curves of amino acids from a hydrolysate of polymyxin B_1.

Molecular Weight Studies.—The method of partial substitution[8] was used: 160 mg. of polymyxin B_1 dissolved in 5 ml. of ethanol, 75 mg. of $NaHCO_3$ dissolved in 25 ml. of H_2O and 75 mg. of fluoro-2,4-dinitrobenzene dissolved in 25 ml. of ethanol were mixed at 25° in the dark. The reaction was followed spectrophotometrically by periodic readings at 350 mμ. The same reaction mixture without the fluoro reagent was used as a blank. After 20 minutes an optical density per cm. of about 30 was reached, which in theory corresponded roughly to that of the mono dinitrophenyl (DNP) derivative of the peptide. The reaction was interrupted at this point by addition of glacial acetic acid. The yellow solution was concentrated to about 5 ml. at 25° and 9 mm. pressure and extracted three times with 10-ml. portions of ethyl ether. After evaporation to dryness the residue was distributed in the system 2-butanol–0.1 *N* HCl. This is the system used previously for purifying polymyxin B. Analyses were made by weight and by ultraviolet absorption at 350 mμ after 198 transfers had been applied. An interpretation of the result shown in Fig. 5 will be given in the Discussion.

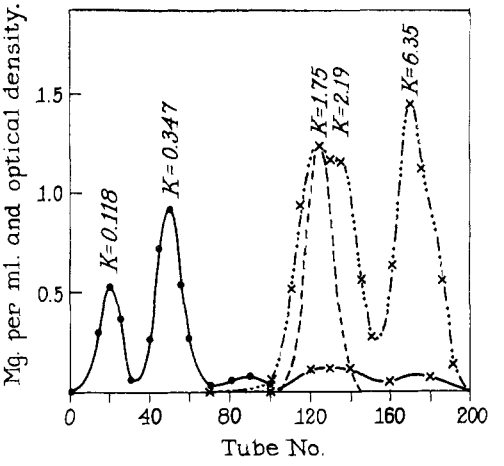


Fig. 5.—Distribution curves of partial DNP-substitution products of polymyxin B_1: system, 2-butanol–0.1 *N* HCl; phases, 10 ml. upper, 10 ml. lower; charge, in tube 1; transfers, 198; ●—●, lower phase; ×—×, upper phase; ×---×, optical density at 350 mμ of upper phase; ---, theoretical curve.

Discussion

Hydrolytic studies with polypeptin indicated difficulty in completely removing the organic acid from the aqueous phase. For this reason it seemed advisable to use a system for countercurrent distribution of the acid which was completely volatile, rather than one containing phosphate buffers.[9] Since buffers are known to improve selectivity, the use of pyridine as a component in the system was tried. A system made from *n*-heptane and 20% aqueous pyridine showed high selectivity but did not give a linear partition isotherm. Nevertheless, in spite of skewed distribution bands, it was effective for studying the purity of the acid fraction from hydrolysis of polymyxin B.

In skewed bands such as those in Fig. 1 the calculated theoretical curve cannot be matched and used as a criterion of purity. This difficulty was overcome by isolation of the acid from each band in

(8) A. R. Battersby and L. C. Craig, THIS JOURNAL, **73**, 1887 (1951).

(9) G. T. Barry, Y. Sato and L. C. Craig, *J. Biol. Chem.*, **188**, 299 (1951).

TABLE I

ANALYTICAL DATA OF POLYMYXIN B_1 AND ITS COMPONENTS

	Analyses, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found	Calcd.	Found	Optical rotation Literature	Found $[\alpha]^{25}D$
Polymyxin $B_1 \cdot 5HCl$ $C_{56}H_{104}O_{14}N_{16}Cl_5$	47.9	47.7	7.5	7.4	16.0	15.9	Cl: 12.65	12.9	——	$-85.1°$ (c 2.33 in 75% ethanol in H_2O)
α,γ-Diaminobutyric acid, monohydrochloride	31.1	31.1	7.2	7.0	18.1	18.4[a]			$[\alpha]^{25}D$ $+31.6°$[b] (c 0.5–2 in 5 N HCl)	$+20.3°$ (c 2.07 in 6 N HCl)
L-Threonine	40.3	40.3	7.6	7.5	11.8	11.9			$[\alpha]^{26}D$ $-28.3°$[c] (c 1.1 in H_2O)	$-29.2°$ (c 0.357 in H_2O)
L-Leucine	54.9	55.0	10.0	10.0	10.7	10.7			$[\alpha]^{26}D$ $+22.5°$[b] (c 0.5–2.0 in CH_3COOH)	$+22.1°$ (c 1.178 in CH_3COOH)
D-Phenylalanine	65.4	65.2	6.7	6.6	8.5	8.3			$[\alpha]^{20}D$ $+35.0°$[c] (c 2.04 in H_2O)	$+33.6°$ (c 0.714 in H_2O)
Acid 1, $C_9H_{18}O_2$							Ag:		Na salt	
Silver salt	40.8	40.7	6.5	6.4			40.7	41.1	——	$+7.0°$
Amide	68.8	68.8	12.2	12.0	8.9	8.9				(c 2.0 in H_2O)
Acid 2, $C_8H_{16}O_2$							Ag:		Na salt	
Silver salt	38.2	38.2	6.0	5.8			42.9	42.5	——	$0.0°$
Amide	67.2	66.9	11.9	11.7	9.8	9.7				(c 2.0 in H_2O)

[a] Burned slowly. [b] J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *J. Biol. Chem.*, **204**, 307 (1953). [c] "Merck Index," 6th Edition.

two cuts and study of each cut separately by preparation of crystalline derivatives. No difference between the two cuts from each band could be found and only two acids in the original hydrolysate are therefore indicated.

Countercurrent distribution of the intact polypeptide preparation clearly revealed the presence of more than one peptide. Hydrolysis of one of these, here called polymyxin B_1, followed by isolation of the organic acid fraction and countercurrent distribution showed that B_1 contained only the C_9 acid. The C_8 acid must therefore be present in the other peptide of the mixture, polymyxin B_2.

Molecular weight studies of polymyxin B_1 by the method of partial substitution gave a distribution pattern which appeared clearly interpretable. In Fig. 5 the first band from the left was found to contain inorganic salt. The second band was found to contain unsubstituted polypeptide. The third band was yellow in color and from the reasoning given in ref. 8 should contain one or more of the mono-substituted derivatives. That more than one was present was indicated by the shoulder in the band and by the fact that it was definitely broader than the calculated band for a single substance. However, the weight extinction ratios at 350 mμ throughout the band were constant. Further distribution of this band failed to reveal the presence of a solute with a weight extinction ratio different from that noted in Fig. 5, but did further separate the mixture of isomers. Since for molecular weight calculation a mixture of monosubstituted derivatives would give the same result as a single pure monosubstituted derivative the fractionation was not carried further. The weight extinction data, $D = 9.89$ per mg. per ml., permitted calculation[8] of a molecular weight of 1150 $\pm$ 10% for the free base.

The shape and position of the fourth band with a K of 6.35 indicated it to be a mixture of higher substitution products. This was confirmed by the weight density ratios throughout the band. It was not studied further.

Absorption spectrum studies with polymyxin B_1 in the ultraviolet region indicated only the type of absorption which would be caused by a phenylalanine residue. The weight extinction ratio at 259 mμ indicated a minimum molecular weight approximating 1100.

Complete hydrolysis of polymyxin B_1 and fractionation of the products gave a clear analytical picture. The peptide contains α,γ-diaminobutyric acid, threonine, leucine, phenylalanine and 6-methyloctan-1-oic acid. No other residue is present. The identity of the amino acid fragments was established by the analytical data given in Table I and by paper chromatography. Derivatives of the fatty acid were prepared. Melting point, infrared spectrum and analytical data were used to identify the acid.

The weight patterns in Fig. 4 permit calculation of the number of residues except for one uncertainty. This concerns the amount of HCl present in a residue when an aliquot of the system is evaporated. This uncertainty did not concern the leucine and phenylalanine bands since in the final distribution a neutral system was used. For a more quantitative estimate of the amount of α,γ-diaminobutyric acid and threonine the whole bands were separately removed, evaporated and the residue dissolved in a standard volume of water. A Kjeldahl analysis on an aliquot of the first amino acid gave the total amount. The amount of threonine was derived from a quantitative ninhydrin determination[10] on the standard solution. The results indicated 6, 2, 1, 1 moles $\pm$ 5%, respectively, for α,γ-diaminobutyric acid, threonine, leucine and phenylalanine.

(10) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

Assuming the single fatty acid residue to be bound to one of the amino groups and the presence of a single free carboxyl group as indicated by the titration curve,[11] the amino acids given above can be joined to give an empirical formula $C_{56}H_{99}O_{14}N_{16}$. This corresponds to a molecular weight of 1220. Analytical data obtained on the hydrochloride are in agreement with this formula.

Optical rotations were taken on the residues obtained by evaporation of the solvents directly from the distribution shown in Fig. 4 and again after recrystallizing the residues. In the case of leucine and phenylalanine no change in rotation was noted after crystallization. The phenylalanine was of the D-configuration. With threonine the rotation of the residue was lower than expected and indicated a slight degree of racemization during hydrolysis. The exact amount is somewhat uncertain because the chloride content of the residue was not determined. When converted to the free amino acid and crystallized, the full rotation was noted.

In the case of α,γ-diaminobutyric acid the rotation of the residue obtained directly from the distribution indicated nearly one third to be racemic, or perhaps one of the six residues to be of the D-configuration. After recrystallization the rotation was only very little higher and still roughly two thirds of that of the L form.

The next step in the study of the structure of this peptide will involve partial hydrolysis in order to determine the sequence of the amino acids. This is being undertaken.

The authors wish to thank Dr. H. Jaffe for his assistance in the spectral studies. We wish to acknowledge with appreciation the technical assistance of Miss E. A. Jacobs and Miss G. Walker. The elementary analyses were carried out by Mr. D. Rigakos and Mr. T. Bella.

NEW YORK, N. Y.

(11) T. S. G. Jones, *Ann. N. Y. Acad. Sci.*, **51**, 909 (1949).

[CONTRIBUTION FROM THE LABORATORY OF CHEMICAL PHARMACOLOGY, NATIONAL CANCER INSTITUTE[1]]

The Structure of Savinin

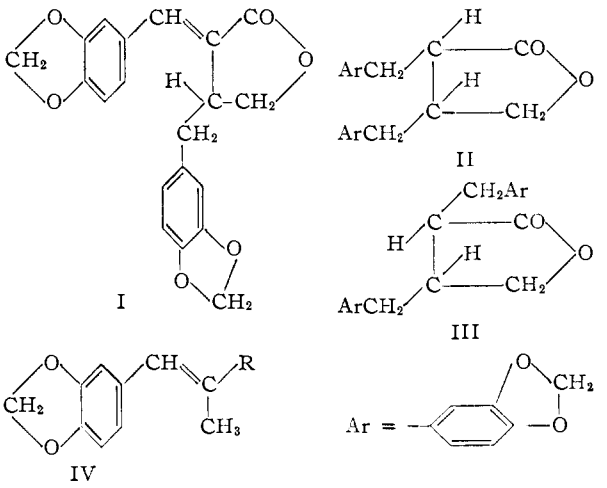
BY ANTHONY W. SCHRECKER AND JONATHAN L. HARTWELL

RECEIVED APRIL 7, 1954

The structure of savinin (I) has been established by its hydrogenation to (+)-isohinokinin (II) and by spectroscopic evidence of its α,β-unsaturated lactone nature. A pronounced bathochromic effect in the ultraviolet spectra of lactones of the savinin type is discussed.

In addition to podophyllotoxin, a compound devoid of tumor-damaging potency was isolated from the dried needles of *Juniperus sabina* and named savinin.[2] Although it had been recrystallized to a constant melting point of 146–148°, the presence of some contaminant was indicated by its analysis.[2] Further purification by chromatography, followed by additional recrystallizations and prolonged drying *in vacuo* has now provided material which still melted at 146.2–147.3°, but now gave analytical figures agreeing closely with the empirical formula $C_{20}H_{16}O_6$. Catalytic hydrogenation afforded dihydrosavinin, $C_{20}H_{18}O_6$, which appeared to be identical with (+)-isohinokinin[3] (II) by its optical rotation and melting point.[4] This was established by base-catalyzed epimerization to (−)-hinokinin[3,5] (III), the identity of which was proved by mixed melting point determination and comparison of infrared spectra.[6]

Several structural formulas for savinin are consistent with its optical activity and its hydrogenation to (+)-isohinokinin. A choice can be made on the basis of the ultraviolet and infrared spectra, which demonstrate that savinin must possess the α,β-unsaturated lactone structure I and exclude alternative β,γ-unsaturated lactone formulations. Thus the ultraviolet absorption spectrum of savinin is characteristic of that of 3,4-methylenedioxycinnamic acid derivatives.[7,8] This is illustrated by comparison of its spectrum with those of methyl α-methyl-3,4-methylenedioxycinnamate (IV, R = $-CO_2CH_3$) and of α-methyl-3,4-methylenedioxycinnamyl acetate (IV, R = $-CH_2OCOCH_3$) (Fig. 1). The infrared spectra (Fig. 2) of both savinin



(1) National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare.

(2) J. L. Hartwell, J. M. Johnson, D. B. Fitzgerald and M. Belkin, THIS JOURNAL, **75**, 235 (1953).

(3) S. Keimatsu and T. Ishiguro, *J. Pharm. Soc. Japan*, **56**, 103, 901 (1936) (German summaries: pp. 19, 187).

(4) Dihydrosavinin was first obtained in the form of needles, m.p. 106–107°, unchanged after recrystallization, while subsequent experiments provided leaflets, m.p. 116.3–116.7° (lit.[3] plates, m.p. 116–117°). Chloroform solutions of these polymorphic modifications had identical infrared spectra and optical rotations.

(5) R. D. Haworth and D. Woodcock, *J. Chem. Soc.*, 1985 (1938).

(6) We are indebted to Prof. R. D. Haworth for an authentic sample of (−)-hinokinin.

(7) R. Mendes da Costa, *Compt. rend.*, **196**, 1815 (1933).

(8) I. A. Pearl and D. L. Beyer, *J. Org. Chem.*, **16**, 216 (1951).