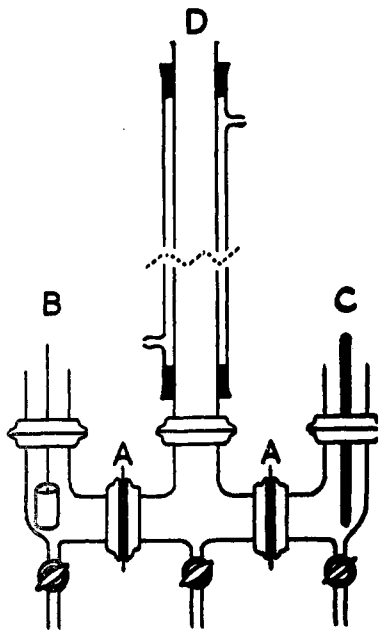

Fig. 3.—Electrodecantation apparatus: A, cellophane membranes; B, platinum anode; C, carbon cathode; D, central compartment containing solution to be electrodecanted.

Pauly diazo test as modified by MacPherson[19] for histidine. The equivalent N-acetylglucosamine content was determined as described previously.[11]

Buffer Capacity.—Approximately 50 mg. of a preparation was dissolved in 5 ml. of distilled water and 0.1 *N* HCl added until the *p*H was approximately 3. The solutions were then titrated with 0.0326 *N* NaOH with the aid of a Beckman *p*H meter. A control consisting of 5 ml. of distilled water was titrated similarly.

Fractionation of Gastric Mucin.—The procedure employed is diagramed in Fig. 1. Ethanol concentrations are volume per cent. and all solutions were stored at 5° between operations. In general all dialysis operations were conducted at 5° for at least five days against distilled water which was changed frequently. The yields of the various fractions were not corrected for the 6% of water present in the Wilson mucin.

The electrodecantations were conducted in an apparatus constructed from standard 2-inch Pyrex pipe fittings (Fig. 3). The center compartment including the four-foot length of standard 2-inch Pyrex pipe had capacity of 2500 ml. A limit potential of 700 volts was used and the anode and cathode compartments were maintained at 25–30° both by controlling the potential and by circulating cold water through glass coils contained in these compartments. These cooling coils are not shown in Fig. 3. With this apparatus, separation of insoluble material occurred after a lapse of about forty-five to sixty minutes eventually leaving a fairly clear supernatant zone contained for the most part in the long central arm. This fractionation, beginning at the upper surface of the central arm, is probably due to circulation currents established in the solution after a short period of operation. The circulation occurred below the boundary between the turbid and supernatant zones and on the cathode side of the column was upward and on the anode side, downward. The liquid moving upward was fairly clear and appeared to layer on top of the boundary. At the same time considerable solid accumulated in the lower part of the cell and against the anode membrane. The solution was conveniently separated into a fairly clear upper layer, a slightly turbid central zone, and a viscous residue. The migration and precipitation of non-dialyzable, negatively charged material at the anode membrane and the resulting *p*H changes in solution are probably important in establishing the observed circulation currents. Occasional turbulence of the boundary during electrodecantation was minimized by protecting the column from uneven external heating by means of a water jacket.

Acknowledgment.—The authors wish to express their indebtedness to Dr. W. T. J. Morgan for the data given in Table IV and to Dr. D. H. Brown and Dr. E. L. Bennett for their assistance during the course of this investigation.

Summary

By a combination of ethanol fractionation and electrodecantation an A-substance preparation has been isolated from hog gastric mucin. This preparation has greater activity in respect to inhibition of hemolysis and inhibition of isoagglutination than preparations hitherto derived from this source.

PASADENA, CALIFORNIA RECEIVED MAY 12, 1948

(19) H. T. MacPherson, *Biochem. J.*, **36**, 59 (1942).

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 1219]

The Isolation of Two Forms of Blood Group A-Substance from Hog Gastric Mucin[1]

BY GEORGE HOLZMAN[2] AND CARL NIEMANN

A systematic study of the effect of *p*H and ionic strength upon the turbidity of aqueous solutions of two potent A-substance preparations obtained from commercial (Wilson) hog gastric mucin by ethanol fractionation and electrodecantation[3] has shown (Fig. 1) that maximum separation of a solid phase occurs in the region of *p*H 3.0–3.5 in a solution of minimum ionic strength and that each of the above preparations may be separated, in solutions 0.0011 *F* in hydrochloric acid, into acid-insoluble and acid-soluble fractions. The procedure used for the isolation of these two fractions is based upon fractionation in aqueous media of low ionic strength and thus differs from previous methods of fractionation dependent upon the use of organic solvents[3,4,5] or high salt concentrations.[5,6] The separation of water-insoluble fractions has been noted previously in connection with the electro-

(1) This work was supported in part by a grant from the U. S. Public Health Service.

(2) Allied Chemical and Dye Corporation Fellow 1946–1947; present address, Shell Development Co., Emeryville, California.

(3) G. Holzman and C. Niemann, THIS JOURNAL, **72**, 2044 (1950).

(4) K. Landsteiner and R. A. Harte, *J. Exptl. Med.*, **71**, 551 (1940).

(5) W. T. J. Morgan and H. K. King, *Biochem. J.*, **37**, 640 (1943).

(6) C. A. Zittle, *Arch. Biochem.*, **17**, 195 (1948).

decantation of aqueous solutions of mucin,[7,8] and partially purified A-substance preparations derived from this source[3,8] and in the treatment of such solutions with ion-exchange resins.[9] However, these water-insoluble fractions were always less active than the corresponding water-soluble fractions. At present it is not known whether the acid-soluble and acid-insoluble forms of A-substance obtained in this study are to be compared with the water-soluble and water-insoluble A-substance preparations of Morgan and King[5] obtained by heating a water-soluble A-substance preparation with dilute acetic acid, followed by precipitation with ammonium sulfate and subsequent dialysis of the precipitate.

The solubility of the acid-insoluble components of fractions R18-F10 and R18-F11[3] in 0.0011 *F* hydrochloric acid is less than 100 mg. per liter. However their solubility in this solvent can be increased to a value greater than 5 g. per liter if sufficient salt is added to increase the ionic strength of the solution to 0.0109. These unique solubility properties are not altered when the acid-insoluble components are treated with crystalline pepsin at *p*H 2.2 to 2.3. The approximate solubility of the acid-insoluble fraction R20-F2 in 0.0011 *F* hydrochloric acid at 25° was estimated by adjusting aqueous solutions containing varying amounts of this preparation to *p*H 3.0, noting the presence or absence of a solid phase,[10] centrifuging these

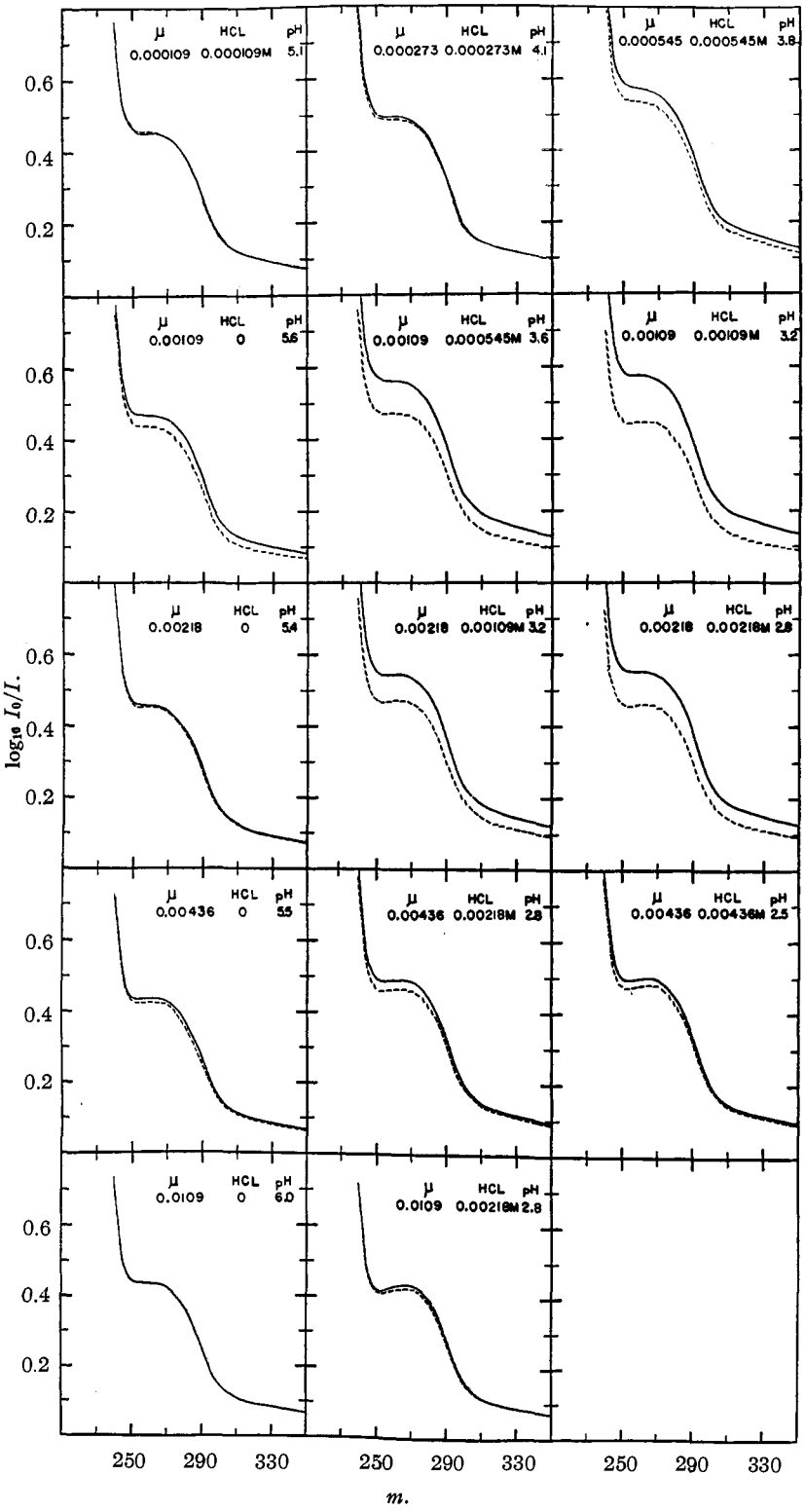

Fig. 1.—Extinction curves of an A-substance preparation before and after separation of insoluble components: —, spectra of solutions immediately after adjustment of *p*H and ionic strength; - - -, spectra of supernatant after standing and centrifugation.

(7) G. Holzman, E. L. Bennett, D. H. Brown and C. Niemann, *Arch. Biochem.*, **11**, 415 (1946).

(8) Holzman, *et al.*, *ibid.*, **13**, 421 (1947).

(9) E. L. Bennett and C. Niemann, *J. Biol. Chem.*, **176**, 969 (1948).

(10) A detectable turbidity on acidification was observed to occur at a concentration of 0.046 mg. fraction R20-F2 per ml. but a visible precipitate was detectable only at 0.092 mg. per ml.

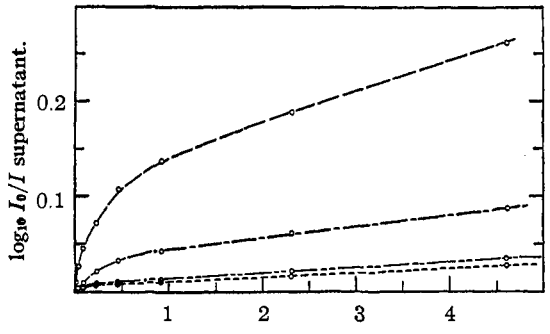

Fig. 2.—Solubility of Fraction R20-F2 in 0.0011 *F* hydrochloric acid at 25°: ——, densities at 220 mμ; — — —, densities at 230 mμ; — — — —, densities at 240 mμ; – – – – –, densities at 260 mμ.

solutions, and determining the extinction curves of the supernatant liquids. In Fig. 2 the extinction values at various wave lengths are plotted as a function of the total amount of solid added. Although these curves show typical breaks at the occurrence of a solid phase the fact that the extinction values increase with increasing amounts of solid phase clearly demonstrates that fraction R20-F2, the most active A-substance preparation which we have obtained from hog gastric mucin, is inhomogeneous with reference to components absorbing in the ultraviolet. The acid-soluble fraction R20-F1 is obviously contaminated with a small amount of the acid-insoluble fraction R20-F2; the fact that the former preparation appears to be electrophoretically homogeneous (Table I) as well as the fact that the two fractions are obtainable from fraction R18-F11 which is itself electrophoretically homogeneous[2] only serves to illustrate the inadequacy of this technique for establishing the homogeneity or inhomogeneity of many A-substance preparations. However in the case of the acid-insoluble fraction R20-F2 electrophoretic analysis has disclosed the presence of two components (Table I) and it is estimated that this fraction contains approximately 15% of the fast moving component. The other known chemical and physical properties of the acid-soluble fraction R20-F1 and the acid-insoluble fraction R20-F2 are given in Table I and Fig. 3. None of these data suggest a reason for the striking difference in the solubilities of fractions R20-F1 and R20-F2 and in general the constants that have been observed are those that would be expected of highly purified and undegraded A-substance preparations derived from hog gastric mucin.[3]

The acid-soluble fractions R19-F1 and R20-F1 and the acid-insoluble fractions R19-F2 and R20-F2 possess equivalent activity within the limits of experimental error in inhibiting the isoagglutination of human A-cells (Table II). In the more precise test based upon the inhibition of hemolysis of sheep cells by human A-cell immune rabbit sera the acid-insoluble fractions appeared to be more active than the soluble fractions though the difference was not great and was just within the extreme limits of experimental error (Table II).

TABLE I

CHEMICAL AND PHYSICAL PROPERTIES OF A-SUBSTANCE PREPARATIONS

		Acid-soluble component R20-F1	Acid-insoluble component R20-F2
Carbon %		41.1	42.6
Hydrogen %		7.2	7.4
Nitrogen % (Dumas)		5.1	5.6
Ash % (residue on combustion)		0.8	0.5
Equivalent arginine % (before hydrolysis)		0.30	0.29
Equivalent histidine % (before hydrolysis)		0.48	0.53
Maximum tyrosine % (from spectra)		1.0	0.8
Maximum tryptophan % (from spectra)		0.3	0.2
Equivalent N-acetyl-D-glucosamine %		13.0	11.5
Equivalent D-galactose %		31	26
Base-combining capacity from *p*H 3 to 8, moles × 10^5 per gram		11	10
Acid-combining capacity from *p*H 8 to 10.5, moles × 10^5 per gram		4	7
Extinction coefficients, citrate–phosphate buffer at *p*H, 7.3 $E^{1\%}_{1cm.}$	at 260 mμ	1.17	0.87
	at 350 mμ	0.13	.14
Optical rotation, 1% in phosphate buffer, *p*H 8.0; μ 0.136, $[\alpha]^{25}D$		+1 ± 2°	+19 ± 2°
Electrophoretic mobility, acetate buffer, *p*H 3.85, μ 0.132, 1.35°, cm.^{2} volt^{-1} sec.$^{-1}$ × 10^5, anodic,	main component	1.1	1.1
	fast component	...	3.0

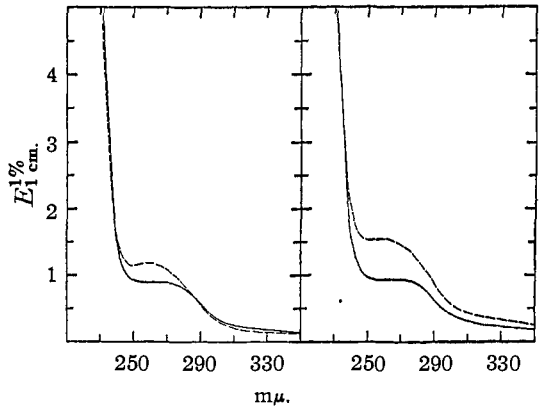

Fig. 3.—Extinction curves of the acid-soluble and acid-insoluble components in citrate phosphate buffer *p*H 7.3: left, – – – –, R20-F1; —, R20-F2; right, – – – –, R19-F1; —, R19-F2.

The parallel behavior of the acid-soluble and insoluble fractions in the isoagglutination and hemolysis tests indicates that these preparations are undegraded, relative to gastric mucin, since a diminution in activity in the isoagglutination test with retention of activity in the hemolysis test is ordinarily associated with the behavior of degraded preparations.[4,5,8] It should be pointed out that there is no evidence that the acid-soluble and acid-insoluble components obtained in this study are related to the two forms of A-substance isolated from human urine by Jorpes and Thaning,[11] one of which inhibited isoagglutination of human A cells and the other the lysis of sheep cells by human A-cell immune rabbit sera.

The acid-soluble and acid-insoluble fractions have been examined by Dr. W. T. J. Morgan in respect to their abilities to inhibit the agglutination of human O-cells by selected cattle sera (Table III). While the acid-insoluble component appears to be purer than the acid-soluble component with respect to contamination by H-substance, the precision of the agglutination test is such that the difference may not be significant. However, the four-fold increase in A-titer and the four-fold decrease in H-titer in going from the water-soluble solids of gastric mucin to the acid-insoluble component R19-F2A would appear to lend support to the contention[3] that a mixture of A- and H-substances is separable.

TABLE II

SEROLOGICAL PROPERTIES OF A-SUBSTANCE PREPARATIONS OBTAINED FROM HOG GASTRIC MUCIN

Preparation	Inhibition of isoagglutination titer[a,b]	Inhibition of hemolysis titer[b,c]
R18-F10[3]	150 ± 30	0.065 ± 0.005
R18-F11[3]	150 ± 30	.062 ± 0.005
R19-F1	130 ± 40	.063 ± 0.005
R20-F1	150 ± 40	.060 ± 0.005
R19-F2	160 ± 40	.055 ± 0.005
R20-F2	185 ± 40	.051 ± 0.005

[a] Microliters group B serum neutralized per microgram substance. [b] Data reported are the mean of duplicate or triplicate analyses with indicated average deviation. [c] Micrograms substance inhibiting 50% hemolysis.

TABLE III

SEROLOGICAL SPECIFICITY OF A-SUBSTANCE PREPARATIONS[a]

Fraction	Agglutination inhibition titers, dilution of fraction inhibiting: Anti-H × 10³	Anti-A × 10³
C-135 (water-soluble solids of mucin)	1:1280	1:320
R18-F11	1:640	1:640
R19-F1A (acid-soluble component)	1:640	1:640
R19-F2A (acid-insoluble component)	1:320	1:1280

[a] For details see ref. 3.

In recent years Kabat[12–14] has advocated the use of a quantitative precipitin test for determining the relative purity[13] as well as the "absolute" purity[14] of A-substance preparations, the latter being based upon the estimation of the proportion of hexosamine present in a preparation precipitable by an excess of anti-A sera. With respect to the latter procedure it was recognized[14] that aside from the uncertainties introduced by the use of normal human γ-globulin as a reference standard and an inhomogeneous antibody, an additional uncertainty was introduced by a significant solubility correction (10–20%) based upon the assumption that only a single substance precipitates. It is difficult to reconcile this latter assumption with the fact that two forms of A-substance can be isolated from hog gastric mucin and for the present one cannot accept with confidence, estimates of the "absolute" purity of A-substance preparations derived from Wilson hog gastric mucin.

Experimental

Analytical Methods.—The nature and limitations of most of the analytical methods used in this study have been discussed previously.[3,7,8,15] The values given for equivalent per cent. galactose were obtained by a modification[16] of the carbazole procedure of Gurin and Hood[17] and are useful for comparative purposes only.

A-Substance Preparations.—The most active fractions, *i. e.*, R18-F10 and R18-F11, obtained from hog gastric mucin by ethanol fractionation and electrodecantation[3] were used as starting materials in these studies. These fractions were similar in respect to their chemical, physical and serological properties.

Fractionation of Preparation R18-F10.—The apparent extinction coefficients[15] of aqueous solutions containing 4.62 mg./ml. of fraction R18-F10 and varying amounts of hydrochloric acid and sodium chloride are given in Fig. 1. Using these data as a guide 1.0 ml. of 0.012 *F* hydrochloric acid was added to 10 ml. of water containing 50.8 mg. of fraction R18-F10 and the solution (*p*H 3.2) allowed to stand overnight at 5°. The gelatinous precipitate, estimated to represent 15% of the weight of the starting material, which was collected by centrifugation in an ordinary laboratory type centrifuge was readily soluble in distilled water and acid solutions containing sodium or barium chloride but relatively insoluble in 0.00109 *F* hydrochloric acid.

Fractionation of Preparation R18-F11.—To one liter of water containing 5.59 g. of fraction R18-F11 was added 100 ml. 0.012 *F* hydrochloric acid, the turbid solution (*p*H 3.1) centrifuged, the supernatant liquid decanted and lyophilized to give 4.25 g. (76%) of fraction R19-F1. A portion of fraction R19-F1 dissolved in distilled water was dialyzed against the same solvent to give fraction R19-F1A. The precipitate was washed four times, by resuspension and centrifugation, with 110 ml. of 0.00109 *F* hydrochloric acid, the residue taken up in 50 ml. of distilled water and lyophilized to give 0.83 g. (15%) of fraction R19-F2. The four solutions obtained in the washing process were lyophilized separately and were found to contain, from the first to the fourth washing, 150, 52, 34 and 28 mg. of solid, respectively. A portion of fraction R19-F2 was dialyzed against distilled water to give fraction R19-F2A.

In a second experiment the acid-insoluble component was reprecipitated rather than washed. The insoluble material separating from 3.3 liters of 0.00112 *F* hydrochloric acid containing 21.3 g. of fraction R18-F11 was collected by centrifugation, the supernatant liquid de-

(11) E. Jorpes and T. Thaning, *J. Immunol.*, **51**, 221 (1945).

(12) A. Bendich, E. A. Kabat and A. Bezer, THIS JOURNAL, **69**, 2163 (1947).

(13) Bendich, *et al.*, *J. Exptl. Med.*, **83**, 477 (1946).

(14) Bendich, *et al.*, *ibid.*, **83**, 485 (1946).

(15) G. Holzman and C. Niemann, *J. Biol. Chem.*, **174**, 305 (1948).

(16) G. Holzman, R. V. MacAllister and C. Niemann, *ibid.*, **171**, 27 (1947).

(17) S. Gurin and D. B. Hood, *ibid.*, **131**, 211 (1939).

canted, the latter dialyzed for five days at 5° against distilled water and lyophilized to give 14.1 g. (66%) of fraction R20-F1. The precipitate was reprecipitated five times by dissolving the precipitate in 500 ml. of water, adding 50 ml. of 0.012 *F* hydrochloric acid and centrifuging. The solutions in every case were 0.0011 *F* in hydrochloric acid with the *p*H varying between 3.0 to 3.1. The precipitate obtained after the fifth reprecipitation was dissolved in distilled water, the solution dialyzed for four days at 5° against the same solvent, filtered to remove a few suspended particles and lyophilized to give 3.0 g. (14%) of fraction R20-F2.

Acknowledgment.—The authors wish to express their indebtedness to Dr. W. T. J. Morgan for the data given in Table III and to Dr. D. H. Brown and Dr. E. L. Bennett for their assistance during the course of this investigation.

Summary

The isolation of two forms of A-substance, one soluble and the other insoluble in aqueous solutions at *p*H 3 and low ionic strength, from hog gastric mucin is described.

PASADENA, CALIFORNIA RECEIVED JULY 7, 1948

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF WISCONSIN]

Precursors for Aliphatic Penicillins[1]

BY J. A. THORN AND M. J. JOHNSON

Penicillins so far listed in the literature include both aromatic and aliphatic types.[2] Exclusive of the biosynthetic penicillins described by Behrens, *et al.*,[3] the aromatic penicillins are benzylpenicillin (G) and *p*-hydroxybenzylpenicillin (X). Aliphatic penicillins include 2-pentenylpenicillin (F), *n*-amylpenicillin (dihydro F) and *n*-heptylpenicillin (K). Flavicidin, once reported to be 3-pentenylpenicillin, is now considered to be *n*-amylpenicillin.[4]

Introduction of the use of precursors stimulated considerable research on the problem of producing different penicillins, and many new ones were obtained by the Lilly group under Behrens.[3] Among the types of compounds effectively used as precursors were substituted mercaptoacetic, hydroxyacetic, polycyclic acetic, heterocyclic acetic and phenylacetic acid derivatives. Biosynthesis of penicillins having *n*-alkyl side-chains has not been reported, although several fatty acids were tested as possible precursors.[5,6]

As pointed out by Behrens, *et al.*,[5] yield stimulation data are not conclusive in testing for the precursor activity of compounds since increased formation of a penicillin (or formation of a new penicillin) can occur without a concurrent increase in total yield. This test was not used in the work to be described. Instead, the precursor effects, if any, of the different compounds examined were ascertained by determining quantitatively each of the penicillins formed in experimental and control broths. Comparison of the values so obtained gave the required information. The filter paper chromatographic method of Karnovsky and Johnson[7] was employed for the penicillin analyses.

Since precursors for the natural aliphatic penicillins had not been described, it was thought of interest to investigate the problem. It appeared reasonable to begin by studying the effects of various triglycerides and fatty acids on penicillin formation.

Experimental

Fermentation Techniques.—*Penicillium chrysogenum* Q176 was used exclusively in the work to be described. All fermentations were conducted at 25°, and the 500-ml. Erlenmeyer flasks containing the inoculated medium were agitated by means of a reciprocating shaker operating at 92 cycles per minute and having a horizontal displacement of four inches.

The inoculum medium employed was that of Jarvis and Johnson,[8] except that the ammonium sulfate was replaced by ammonium phosphate and copper sulfate was omitted. The fermentation medium[9] employed contained in g. per l.: lactose, 20; glucose, 10; ammonium acetate, 4; ammonium lactate, 5; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.02; and Na_2SO_4, 0.5. In each case, the sugars were autoclaved separately and were added to the salt solution immediately before inoculation. In the case of the inoculum medium, the calcium carbonate was also sterilized separately in distilled water. The inoculum medium, 80 ml. per flask, was seeded with a spore suspension prepared by the method of Gailey, *et al.*,[10] and placed on a rotary shaker (270 cycles per minute, and imparting a motion such that all points on each flask described a horizontal circle 2.25 inches in diameter). When a thick vegetative growth was obtained (after about forty to forty-four hours); it was used to inoculate the fermentation medium. The fermentation flasks each contained 100 ml. of medium, to which was added 5 ml. of vegetative inoculum.

Compounds Tested for Precursor Activity.—All compounds, unless otherwise specified, were obtained from Eastman Kodak Co., Rochester, and were not further purified.

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Lederle Laboratories, Pearl River, N. Y., and E. R. Squibb and Sons, New Brunswick, New Jersey.

(2) Wintersteiner, Boon, Carrington, MacCorquodale, Stodola, Wachtel, Coghill, Risser, Philip and Touster, in Clarke, Johnson and Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., chapter 5, 1949.

(3) O. K. Behrens, J. Corse, J. P. Edwards, L. Garrison, R. G. Jones, Q. F. Soper, F. R. Van Abeele and C. W. Whitehead, *J. Biol. Chem.*, **175**, 793 (1948).

(4) M. Adler and O. Wintersteiner, *ibid.*, **176**, 873 (1948).

(5) O. K. Behrens, J. Corse, D. E. Huff, R. G. Jones, Q. F. Soper and C. W. Whitehead, *ibid.*, **175**, 771 (1948).

(6) O. K. Behrens, chapter 19, in ref. 2.

(7) M. L. Karnovsky and M. J. Johnson, *Anal. Chem.*, **21**, 1125 (1949).

(8) F. G. Jarvis and M. J. Johnson, THIS JOURNAL, **69**, 3010 (1947).

(9) M.-S. Wong, B. S. Thesis, University of Wisconsin, 1947.

(10) F. B. Gailey, J. J. Stefaniak, B. H. Olson and M. J. Johnson *J. Bact.*, **52**, 129 (1946).