viously described. From the mother liquor of the 3-methylisoquinolinium bis-salt was obtained a small yield of buff prisms with m. p. identical to that of the mono-salt; the mixed m. p., however, showed a large depression. Nothing further was done with the new product. In the case of the quinoline reaction, only the mono-salt was isolated (in 40% yield). A mixture of bromohydrin and 2.4 moles of α-picoline, heated without solvent on the steam-bath for twenty-three hours, gave a 20% yield of bis-salt and no recoverable mono-salt.

Preparation of Bis-salts from 2,5-Dibromohexane.—It was anticipated that the yields of bis-salts with this secondary bromide would be less than with the previously used primary bromides and this was generally realized. At the end of the usual period of refluxing, there was little evidence of reaction as judged by adding ether and chilling, except in the case of 3-methylisoquinoline. With this exception, the reaction mixtures were freed of their volatile solvents by boiling, and heated on the steam-bath without solvent for seventeen hours. The γ-picoline mixture crystallized on cooling. On the addition of alcohol, and with cooling, the isoquinoline mixture crystallized. After adding alcohol and ether, all the other mixtures crystallized except the α-picoline mixture which deposited an oil requiring several months to crystallize; the γ-picoline mixture also yielded most of its product after treatment with alcohol and ether. The 3-methylisoquinoline (and the quinoline) reaction mixture evolved a strong diene odor during the six and one-half hours of refluxing. After cooling and adding ether, nearly white crystals deposited.

The pyridine product was hygroscopic. An attempt to prepare the iodide by metathesis with potassium iodide in aqueous solution failed. The perchlorate was readily prepared in the usual way.

The α-picoline product could not be obtained constant melting, the m. p. sometimes rising and sometimes lowering after recrystallization. The perchlorate was made in the usual manner but using the crude bromide obtained as an oil from the reaction mixture by adding an excess of ether.

The β-picoline and isoquinoline products behaved normally, were not hygroscopic, and could be purified by crystallization from alcohol–ether and alcohol, respectively.

The γ-picoline product was hygroscopic. The iodide was prepared by metathesis with potassium iodide in concentrated aqueous solution; the desired iodide, only, crystallized out.

The quinoline product was obtained, after recrystallization from alcohol–ether, as hygroscopic, colorless, transparent prisms, melting indefinitely under 100°. A perchlorate was prepared in the usual manner; it melted at 130.2–131.0°. This perchlorate was identical, by analysis and mixed m. p., with an authentic sample of quinoline perchlorate prepared similarly from the components. *Anal.* Calcd. for $C_9H_8ClNO_4$: C, 47.1; H, 3.5; N, 6.10. Found: C, 47.2; H, 3.6; N, 5.9. Quinoline perchlorate has not hitherto been characterized in the literature.[8] The original quinoline product was therefore quinoline hydrobromide. From 0.03 mole of dibromide, 5.45 g. (0.026 mole, if anhydrous) of quinoline hydrobromide was obtained; this would represent a yield of 43% if both bromine atoms were removed from the dibromide.

The 3-methylisoquinoline product proved, by analysis and mixed m. p., to be identical with 3-methylisoquinoline hydrobromide. From 0.03 mole of dibromide, 4.54 g. (0.020 mole) of hydrobromide was obtained.

Summary

1. The synthesis and properties of sixty-one new quaternary ammonium salts, prepared in the course of studies in the chemotherapy of cancer, are reported.

2. The salts were formed by adding alkylene dibromides to heterocyclic bases. In most cases, bis-salts were formed. With glycerol-α,γ-bromohydrin and sterically-hindered bases, mono-salts were formed. In several instances, no addition occurred but the alkylene dibromide lost hydrogen bromide which was isolated as the hydrobromide of the base used.

3. The analysis and m. p. of quinoline perchlorate and of 3-methylisoquinoline hydrobromide are reported.

(8) Lundsgaard, English Patent 163,946 (1921) (*Chem. Zentr.*, **92**, IV, 727 (1921), and Cordier, *Monatsh.*, **43**, 525 (1923) (*Chem. Zentr.*, **94**, II, 948 (1923)) mention the compound but give no properties or analyses.

BETHESDA, MARYLAND RECEIVED AUGUST 8, 1949

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 1202, PASADENA, CALIFORNIA]

The Isolation of Blood Group A-Substance from Hog Gastric Mucin by Ethanol Fractionation and Electrodecantation[1]

BY GEORGE HOLZMAN[2] AND CARL NIEMANN

In a previous communication,[3] wherein it was shown that a modification of the ethanol fractionation procedure of Landsteiner and Harte[4] was preferable to other procedures for the concentration of A-substance from commercial (Wilson) hog gastric mucin, it was noted that further concentration could be expected by a process which was called electrodialysis[3,5,6] but which is now recognized as electrodecantation.[7-9] A method involving both ethanol fractionation and electrodecantation has now been devised and the principal features of its application to the fractionation of hog gastric mucin are given in Fig. 1.

The relative activities of the various fractions, assigning the starting material unit activity, were evaluated on the basis of inhibition of hemolysis[3,10] and inhibition of isoagglutination,[11] the latter test being used primarily to disclose

(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) D. H. Brown, E. L. Bennett, G. Holzman and C. Niemann, *Arch. Biochem.*, **13**, 421 (1947).

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

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

(6) E. L. Bennett and C. Niemann, *ibid.*, **176**, 969 (1948).

(7) M. Adolf and W. Pauli, *Biochem. Z.*, **152**, 360 (1924).

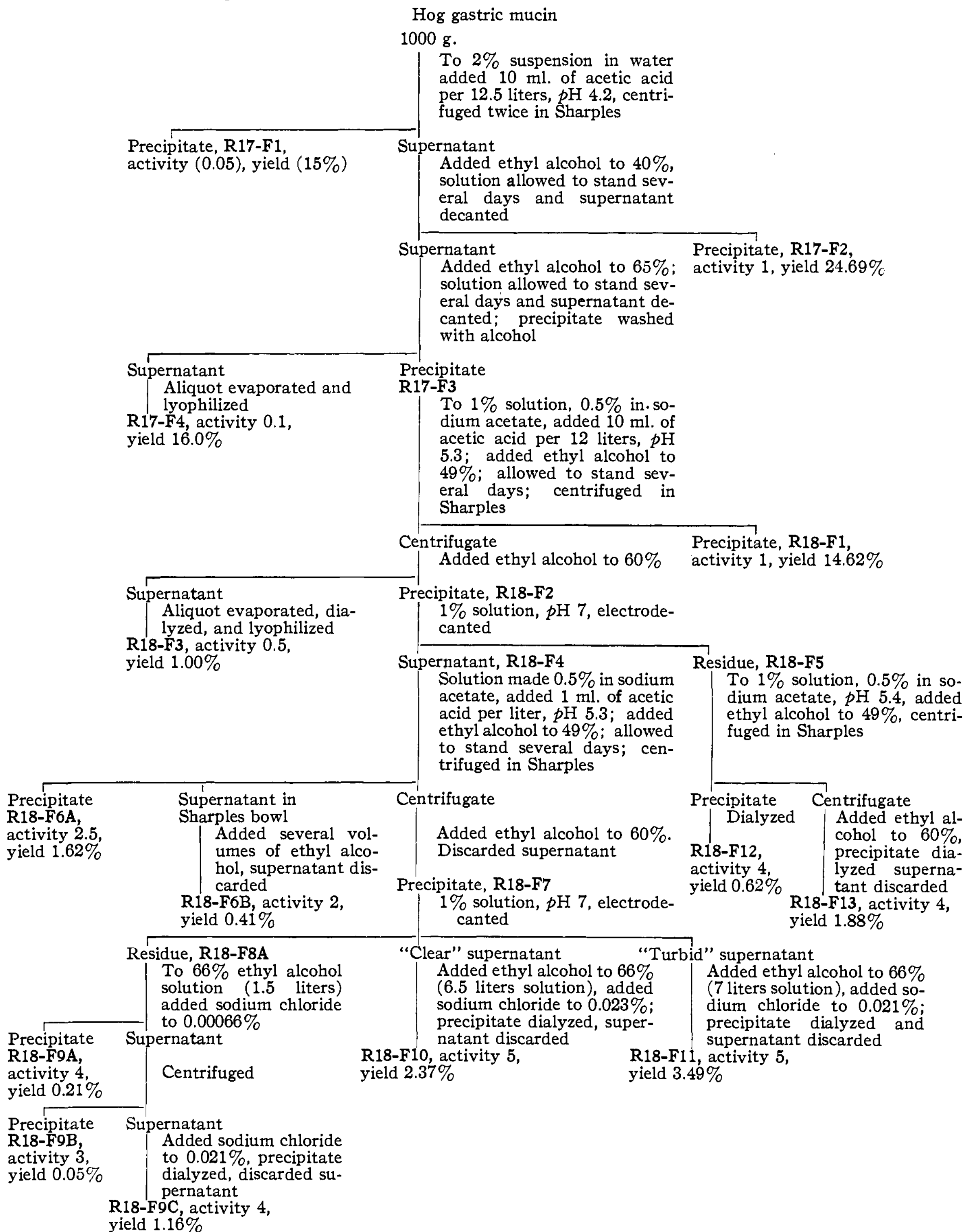
(8) H. Gutfreund, *Biochem. J.*, **37**, 186 (1943).

(9) P. Stamberger, *J. Colloid Science*, **1**, 93 (1946).

(10) D. H. Brown, E. L. Bennett and C. Niemann, *J. Immunol.*, **56**, 1 (1947).

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

Fig. 1.—Isolation of blood group A-substance from hog gastric mucin.



degradation.[3,5] Since no evidence for degradation was obtained, the two tests invariably giving concordant results, the relative activities given in Fig. 1 are believed to be reliable to within the limits of error of the hemolysis test, *i. e.*, ± 10–15%. In Table I the activities of the two most

active fractions, R18-F10 and R18-F11, obtained by the above procedure are compared with the activities of undegraded[3,5] A-substance preparations isolated from hog gastric mucin by other representative procedures.[3]

The extent to which constancy of composition of A-substance has been attained by the new method of isolation is indicated by the data in Table II. Additional data for the two most active fractions are given in Table III. The apparent arginine and histidine contents of the various fractions were determined colorimetrically using unhydrolyzed samples. While low values are usually obtained in this way,[12] the analytical results still have bearing on the extent to which the amino acid composition may be altered by fractionation. It is evident that the color obtained in the Pauly diazo test was in large part due to histidine since the calculated tyrosine content based on this color test for fraction R18-F10, is 2.2%, which is considerably larger than the maximum tyrosine content, 0.7%, found by ultraviolet spectrophotometric analysis.[5] Brand and Saidel[13] have reported that an A-substance preparation obtained by phenol-alcohol fractionation contained 0.6% histidine. The correlation of the equivalent N-acetylglucosamine content with the serological properties of A-substance preparations has been demonstrated previously.[3,11] A similar correlation exists for the fractions described in this communication.

Ultraviolet spectrophotometric analysis has been found to be a sensitive method for following changes in the composition of A-substance preparations.[5] Extinction curves for almost all fractions possess maxima at 260 mμ, and extinction values at this wave length, as well as those at 350 mμ which are indicative of the turbidity of the solution,[5] are given in Table II. In Fig. 2 are shown the successive absorption spectra obtained for the more active preparation in each step of the isolation. In agreement with previous observations,[5] the more active fraction was found to absorb progressively less in the ultraviolet region with each step in the fractionation.

It has been observed[6] that A-substance preparations obtained from hog gastric mucin by a variety of procedures have a small but demonstrable buffering capacity. This observation has been confirmed in this study and in the case of the more active fractions it is estimated on the basis of titrations between *p*H 3 and 10.5 that approximately 16–17 $\times$ 10^{-5} mole per g. of acidic and basic groups are present. This result is not in accord with the observation of Morgan and King[14] that no groups ionizing between *p*H 2 and 10.5 can be detected in A-substance preparations obtained from hog gastric mucin.

(12) A. J. P. Martin and R. L. M. Synge, "Advances in Protein Chemistry," **2**, 56 (1946).

(13) E. Brand and L. J. Saidel, *J. Exptl. Med.*, **83**, 497 (1946).

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

Data obtained by Dr. W. T. J. Morgan in respect to the serological properties of fractions R18-F10 and R18-F11 (Table IV) indicate that in the course of obtaining these fractions from commercial mucin a partial separation of the A- and H-

TABLE I

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

Procedure[a]	No.[a]	Inhibition of isoagglutination titer[b,c]	Inhibition of hemolysis titer[c,d]
Landsteiner–Harte ethanol fractionation	R5-F3, C-143	85 ± 20	0.11 ± 0.01
Morgan–King sodium sulfate fractionation	R2-F2A	45 ± 15	.11 ± 0.01
Morgan–King phenol-ethanol fractionation	C-52	50 ± 15	.13 ± 0.01
Authors ethanol fractionation and electrodecantation	R18-F10	150 ± 30	.065 ± 0.005
	R18-F11	150 ± 30	.062 ± 0.005

[a] For references and details see ref. 3 and 5. [b] Microliters group B serum neutralized per microgram substance. [c] Data reported are the mean of duplicate or triplicate analyses with indicated average deviation. [d] Micrograms substance inhibiting 50% hemolysis.

TABLE II

PROPERTIES OF FRACTIONS OBTAINED ON ETHANOL FRACTIONATION AND ELECTRODECANTATION OF HOG GASTRIC MUCIN

Fraction	Total N	Amino N	Equivalent % arginine	Equivalent % histidine	Equivalent % N-acetylglucosamine	$E^{1\%}_{1 cm.}$ 260 mμ	$E^{1\%}_{1 cm.}$ 350 mμ
Mucin[d]	8.5	1.7	1.6	2.7			
C-135[5]			1.4	2.0	7.6	22.8	0.9
R17-F1	9–10						
R17-F2[d]	8.9	1.0	1.4	1.9	7.5	36.4	1.79
R17-F3[a]	7.1	0.1	0.65	0.99	11.6	9.63	0.77
R17-F4[d]	10.5	4.6	4.3	5.3	0.9	36.4[c]	3.73
R18-F1A[b]	6.8	0.3	0.57	0.93	9.9	8.03	0.99
R18-F1B[b]	7.4	.3	.63	1.07	10.5	9.70	1.22
R18-F1C[b]	7.0	.4	.58	0.99	10.4	8.92	0.84
R18-F2[a]	5.9	.2	.33	0.57	12.5	3.50	0.24
R18-F3	9.1	.8	2.0	1.24	4.0	68.1	4.20
R18-F4[a]	5.0	.1	0.35	0.58	12.5	2.67	0.26
R18-F5	6.0	.1	.32	.55	12.1	5.20	0.33
R18-F6A	6.7	.1	.45	.77	10.9	5.05	1.04
R18-F6B	5.5	.2	.35	.72	12.9	2.47	0.42
R18-F7[a]	4.9	.1	.29	.52	12.3	1.79	.20
R18-F8A	5.5	.1	.27	.50	12.4	2.52	.25
R18-F10[a]	5.0	.1	.29	.56	13.1	0.91	.15
R18-F11[a]	5.2	.1	.29	.53	12.9	1.20	.18
R18-F12	6.2	.1	.38	.70	12.9	3.80	.55
R18-F13	5.3	.1	.28	.50	12.6	2.15	.18
R18-F9A	5.5	.1	.31	.62	12.7	1.36	.26
R18-F9B	6.1	.1	.35	.64	12.0	3.02	.43
R18-F9C	5.6	.1	.25	.48	12.0	1.97	.20

[a] The more active fraction of each step. [b] These fractions correspond to R18-F1. [c] Maximum at 270 mμ, $E^{1\%}_{1cm.} = 38$. [d] Amino acid nitrogen: mucin, 1.3%; R17-F2, 1.0%; R17-F4, 4.4%.

TABLE III

PROPERTIES OF MOST ACTIVE FRACTIONS

	Fraction R18-F10	Fraction R18-F11
Carbon, %	41.6	43.4
Hydrogen, %	7.3	7.3
Nitrogen (Dumas), %	5.0	5.2
Sulfur, %	0.2	0.2
Phosphorus, %	0.04	0.04
Ash (residue on combustion)	0.6	0.6
Base-combining capacity from *p*H 3 to 8, moles $\times 10^5$ per gram	8	9
Acid-combining capacity from *p*H 8 to 10.5, moles $\times 10^5$ per gram	8	8
Optical rotation (c = 1%, phosphate buffer *p*H 7.0, μ 0.136), $[\alpha]^{24}D$	$+7 \pm 2°$	$+3 \pm 2°$
Electrophoretic mobility (acetate buffer *p*H 3.85, μ 0.132, 1.35°), cm.^{2} volt^{-1} sec.$^{-1}$ $\times 10^5$, anodic	1.0	1.1

TABLE IV

COMPARISON OF BLOOD GROUP A AND H ACTIVITIES OF PREPARATIONS FROM HOG GASTRIC MUCIN

Fraction	Agglutination inhibition titer, dilution of preparation inhibiting Anti-A[a] $\times 10^3$	Anti-H[b] $\times 10^3$
C-135[5]	1:320	1:1280
R18-F10	1:640	1:320
R18-F11	1:640	1:640
Lister standard[c]	1:640	1:2560
"H" standard[c]	...	1:5120

[a] Inhibition of isoagglutination of human blood group A cells by serum of individuals of blood group B. [b] Inhibition of agglutination of human O cells by selected cattle sera absorbed with A_1 B cells (see Morgan and Van Heyningen, *Brit. J. Exp. Path.*, 25, 5 (1944); Morgan and Waddell, *ibid.*, 26, 387 (1945); Morgan and Watkins, *ibid.*, 29, 159 (1948)). [c] The Lister standard is an A-substance preparation isolated from hog gastric mucin; the H-substance was isolated from a human pseudomucinous ovarian cyst fluid.

substances,[15] known to be present in this material,[16,17] may have occurred. In view of these observations and those of Aminoff, Morgan and Watkins[16] it is not unreasonable to expect that a homogeneous A-substance preparation will eventually be obtained from commercial mucin.

In the characterization of their A-substance preparations previous investigators have frequently, but not invariably, reported values for "total glucosamine" and "reducing sugar on hydrolysis." We have deliberately abstained, in this and the following communication, from reporting such data because we believe that the methods available for their determination give unreliable and misleading results. The inhomogeneity of A-substance preparations and the concomitant dependence of composition upon the method of preparation demands that an analytical method for the determination of a particular component or group of components, even in a relative sense, at least be specific and free of interferences in respect to the other components that are present in the preparation or its hydrolysate. None of the available methods satisfies these minimum criteria. Furthermore there is no evidence that either of the above values, even if it could be determined accurately, can be correlated with the activity of A-substance preparations derived from hog gastric mucin.

(15) Formerly designated as O-substance, see W. T. J. Morgan and W. M. Watkins, *Brit. J. Expt. Path.*, 29, 159 (1948).

(16) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *Nature*, 158, 879 (1946).

(17) A. Bendich, E. A. Kabat and A. Bezer, *J. Exptl. Med.*, 83, 485 (1946).

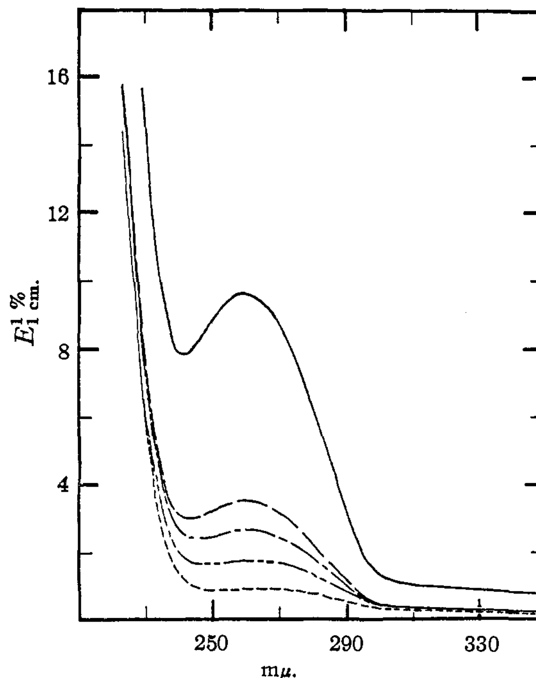


Fig. 2.—Extinction curves of principal fractions obtained on ethanol fractionation and electrodecantation of hog gastric mucin: first ethanol fractionation (R17-F3), —; second ethanol fractionation (R18-F2), — — —; first electrodecantation (R18-F4), — - —; third ethanol fractionation (R18-F7), — - - —; second electrodecantation (R18-F10), - - - - - -.

Experimental

Serological Tests.—The inhibition of isoagglutination and inhibition of hemolysis tests were conducted as described previously.[3,10,11] As before[3,5,6,10,11] when it was necessary to use different lots of reagents the activities of standard preparations were determined coincidentally in order to eliminate deviations due to differences in the reagents.

Absorption Spectra.—The spectra were measured[5] with a Beckman Model DU spectrophotometer equipped with 1-cm. quartz cells using citrate–phosphate buffer at *p*H 7.20 ± 0.05 and A-substance concentrations of approximately 900 micrograms per ml.

Analytical Methods.—Total nitrogen, amino nitrogen, and amino acid nitrogen were determined in duplicate with the aid of the Van Slyke manometric apparatus. Arginine and histidine were determined directly on unhydrolyzed solutions of the various fractions; the Weber modification[18] of the Sakaguchi test was used for arginine and the

(18) C. J. Weber, *J. Biol. Chem.*, 86, 217 (1930).

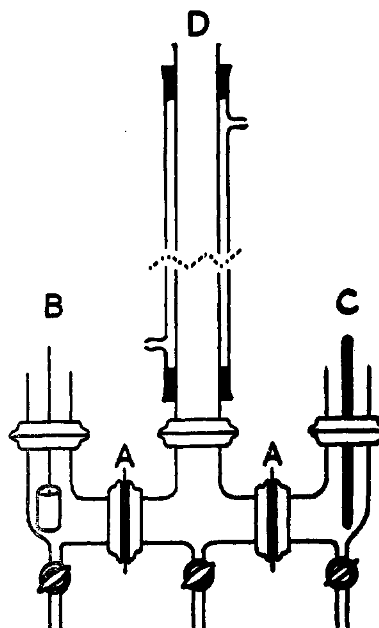


Fig. 3.—Electrodecantation apparatus: A, cellophane membranes; B, platinum anode; C, carbon cathode; D, central compartment containing solution to be electrodecanted.

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).