Studies on Blood Group A Substance

Thesis by

George Holzman

In Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy

California Institute of Technology

Pasadena, California, 1947

Abstract

A study has been made of the isolation of blood group A-substance from various sources with the aim of ascertaining the relative efficacy of procedures in concentrating A-substance. Preparations of A-substance were isolated from hog gastric mucin, hog gastric mucosa, pepsin, human erythrocytes and stromata, and pseudomucinous ovarian cyst fluids according to procedures previously described. This has afforded a direct comparison of the procedures and products obtained.

In order to facilitate the examination of the activities of fractions obtained in isolation procedures, a color test was developed which under certain conditions can be used to predict serological potencies. The carbazole reaction for the estimation of hexoses in polysaccharides was also reinvestigated and an improved procedure developed.

The preparation of A-substance from hog mucin was studied in detail by means of electrodialysis and alcohol fractionation and with the aid of a number of criteria of fractionation including serological potency, elementary analyses, analyses for a few sugars and amino acids, and ultraviolet absorption spectra. Spectrophotometric analyses have also led to the separation of highly potent, electrophoretically homogeneous, preparations into two distinct fractions differing strikingly in their solubility at low ionic strength and low pH. The more active insoluble component was inhomogeneous, however, by electrophoresis, solubility, particle size studies, and serological specificity.

A preliminary investigation of the nature of the alkaline decomposition of A-substance and of some of the products so formed was made.

The diurnal variation in the carbon dioxide content of the extractable gases of leaves of Bryophyllum fedtschenkoi was measured; results were not decisive in testing a hypothesis concerning the mechanism of diurnal acid production of succulents.

Table of Contents page

Studies on Blood Group A Substance

Reprinted from ARCHIVES OF BIOCHEMISTRY
Vol. 11, No. 3, November, 1946
Printed in U. S. A.

A Colorimetric Method for the Estimation of the Activity of Substances Inhibiting the Isoagglutination of Blood Group A Cells[1]

George Holzman, Edward Bennett, David Brown and Carl Niemann

*From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California**

Received July 1, 1946

INTRODUCTION

Investigations on the isolation and characterization of blood group specific substances have relied exclusively on serological tests for the determination of the activities of fractions obtained by various procedures. Isoagglutination inhibition and hemolysis inhibition tests have been used and, more recently, a precipitin test has been developed by Kabat and Bezer (1). In the course of work on the isolation of blood group substances from various sources it was considered advantageous to study a color test which appeared suitable for the estimation of the approximate activity of these preparations. This test has proved useful in our work and is of interest since it emphasizes an intrinsic feature of the structure of A-substance.[2]

The test involves the reaction of Ehrlich's *p*-dimethylaminobenzaldehyde reagent with substances which have been previously treated with a hot alkaline solution; the procedure is essentially that described by Morgan and Elson (2) for the estimation of N-acetylglucosamine. This procedure has been found to give a characteristic red-purple color for A-substance isolated from hog gastric mucin (3) and from human

* Contribution No. 1005.

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

[2] In this paper A-substance refers to material which is capable of inhibiting the isoagglutination of human blood group A cells by serum from individuals of blood group B; evidence is available (5) that some materials which are active in isohemolysis tests may have differing properties.

ovarian cysts (4). The reaction appears to possess some specificity since colors are not given by other polysaccharides containing hexosamine such as chondroitin sulfuric acid and hyaluronic acid (3), or by tryptophan (2), which is known to react with Ehrlich's reagent under other conditions (6).

It has been possible, by means of this reaction, to correlate the serological activities of preparations of A-substance, determined by isoagglutination inhibition tests, with the intensities of colors produced with Ehrlich's reagent. The correlation has been applied to fractions obtained from hog gastric mucin by procedures using mild conditions. The empirical relationship derived between serological activity and color intensity then provides a method for the assessment of the activity of subsequent undegraded A-substance preparations simply from the results of the color test.

While the color test possesses the advantages of considerable convenience and speed and somewhat greater precision than the isoagglutination inhibition test, it is emphasized that the method is not general and must be applied with caution to fractions obtained by procedures involving degradation of A-substance. Furthermore, no attempt has been made to investigate exhaustively all factors influencing the color test, since the limited knowledge of the structure of A-substance inevitably restricts investigations of this kind at present.

Experimental

Color Test

p-Dimethylaminobenzaldehyde (Ehrlich's) Reagent. The reagent was prepared by dissolving 2 g. of Eastman White Label *p*-dimethylaminobenzaldehyde in a solution containing 100 ml. of glacial acetic acid and 2 ml. of 12 *N* HCl. The reagent was stored in bottles protected from light.

Test Solutions of Fractions. Solutions were prepared by dissolving weighed samples in volumetric flasks and diluting with 0.9% NaCl; the test material concentration was usually 1000 γ/ml. It was convenient to use saline as the same solutions could then be used for the serological tests.

N-Acetylglucosamine Solutions. Weighed amounts of dry N-acetylglucosamine were dissolved in 0.9% NaCl, approximately 0.1 saturated with chloroform. Dilute solutions with concentrations in the range 10–100 γ/ml. were prepared by diluting aliquots of more concentrated solutions. It was found that sodium chloride had no significant effect on the color produced with Ehrlich's reagent. N-Acetylglucosamine was prepared essentially according to the procedure of Breuer (7) from *d*-glucosamine hydrochloride. The hydrochloride was neutralized with sodium hydroxide before acetylation.

Procedure for the Analysis of A-substance. One ml. of A-substance solution[3] and 0.1 ml. of 0.25 *M* Na_2CO_3 were added to a 15 ml. calibrated centrifuge tube and the solutions mixed. Two controls were prepared: (I) a blank on the reagents (1 ml. of 0.9% NaCl and 0.1 ml. of 0.25 *M* Na_2CO_3) and (II) a blank on A-substance (1 ml. of A-substance solution and 0.1 ml. of 0.25 *M* Na_2CO_3). The second control was required as A-substance solutions gave a light yellow color after digestion and some solutions gave slight turbidities on final dilutions with acetic acid.

The solutions were heated in a boiling water bath for 15 minutes and cooled by immersion in tap water. The solutions were diluted to 9 ml. with glacial acetic acid and 1 ml. of the *p*-dimethylaminobenzaldehyde reagent added to the A-substance solution and to Control (I). One ml. of glacial acetic acid, 0.24 *N* in HCl (prepared by mixing 1 ml. of 12 *N* HCl and 50 ml. of glacial acetic acid), was added to Control (II). The colors were allowed to develop in a water thermostat at 25.0 ± 0.1°C. for 50–60 minutes. The color intensities were then measured with the Klett-Summerson Colorimeter using a green filter (Klett No. 54); the instrument was preliminarily set at zero against a tube of distilled water. The color intensities were all read in the same Klett tube by rinsing the tube with several portions of solution before adding the major portion. The colorimeter reading for a particular fraction was taken to be equal to the colorimeter reading for the A-substance solution minus the sum of the colorimeter readings for Controls (I) and (II).

Procedure for the Analysis of N-Acetylglucosamine. The procedure was the same as for A-substance except that the digestion with alkali was only for 5 minutes, the time recommended by Morgan and Elson (2). Further digestion with alkali produced very rapid decreases in the intensity of the color with Ehrlich's reagent. Control (II) was not necessary as no turbidities are produced with N-acetylglucosamine in the acetic acid solutions.

Confirmatory and Other Experiments

In the following sections the results of investigations of some of the conditions influencing the color test are summarized.

Conditions of the Alkaline Digestion. One-tenth ml. of 0.25 *M* Na_2CO_3/ml. of A-substance solution gave a maximum color intensity with Ehrlich's reagent with most preparations after 15 minutes digestion at 100°C. About 60–70% of the maximum color intensity was produced after 5 minutes digestion, 90–95% after 10 minutes digestion and 90–100% after 20 minutes digestion. None of the A-substance samples prepared from hog gastric mucin by fractionation with alcohol (8), sodium sulfate (3) or phenol-alcohol (3), from pepsin by autolysis (8) or alcohol fractionation (9), or by treatment of A-substance with papain-HCN or formamide (8) gave any significant color with *p*-dimethylaminobenzaldehyde without prior alkaline digestion.

For a digestion time of 15 minutes, the concentration of sodium carbonate given above (0.1 ml. of 0.25 *M* Na_2CO_3/ml. of A-substance solution) produced the maximum color intensity for an A-substance preparation obtained by sodium sulfate fractionation. The color intensities were 95% and 90% of the maximum color intensity when,

[3] In the present status of the procedure, it is desirable to standardize on a selected concentration of material, *e.g.*, 1000 γ/ml. (± 10%), due to the non-linear dependence of the color on the concentration of A-substance.

respectively, 0.05 ml. and 0.15 ml. of 0.25 *M* Na_2CO_3/ml. of A-substance solution were used. The pH of the solution after digestion with 0.1 ml. of 0.25 *M* Na_2CO_3 was 10–10.5; before digestion the pH was usually somewhat higher, 10.3–10.8. If a test solution is highly buffered, or contains acid, the proper pH will not be maintained during digestion and erroneous results will be obtained.

p-Dimethylaminobenzaldehyde Reagent. The reagent whose preparation is described above had a pale yellow-orange color which increased in intensity on standing; for this reason the reagent was prepared fresh about every 7–10 days. During the period the reagent was being used, the blank color in the analyzed solutions gave very low colorimeter readings in the Klett-Summerson Colorimeter using a green filter (Klett filter No. 54, approximate spectral range, 500–570 mμ). A reagent prepared from *p*-dimethylaminobenzaldehyde recrystallized according to the procedure of Adams and Coleman (10) gave slightly smaller blank colors which increased but little on standing. Furthermore, this reagent, when freshly prepared, gave color intensities for A-substance solutions which were 8–10% greater than those obtained with the unpurified reagent. However, for some reason not thoroughly investigated, this reagent appeared to be somewhat less stable than the reagent prepared from unrecrystallized *p*-dimethylaminobenzaldehyde with respect to the color produced with A-substance; successive analyses of A-substance solutions over a period of several days gave decreasing colorimeter readings. For the purposes of this investigation it was more convenient to use the more reproducible, but somewhat less sensitive reagent prepared from unrecrystallized *p*-dimethylaminobenzaldehyde.

It is apparent from these results that it would be desirable to standardize the procedure so that color intensities produced with A-substance would not reflect changes in the reagent. A few experiments have indicated that N-acetylglucosamine may be a suitable standard as it is stable, can be prepared quite pure, and appears to respond in a similar manner as A-substance to changes in the reagent. Comparisons of the colors obtained with A-substance and N-acetylglucosamine are described in the following sections.

Conditions for the Color Development. The concentration of HCl in the *p*-dimethylaminobenzaldehyde reagent is 0.24 *N;* this differs from the concentration, 0.6 *N* HCl, recommended by Morgan and Elson. The latter concentration was unsuitable; the maximum color intensities for A-substance preparations with the reagent occurred in 10 minutes and the color faded rapidly thereafter at the temperature of development, 25°C. The lower concentration of HCl gave a maximum color intensity in 50–60 minutes for both A-substance and N-acetylglucosamine and the colors were stable for at least 30 minutes longer. The marked effect of temperature on development as well as HCl concentration on the rate of color development was noted by Morgan and Elson, who used temperatures of 13–16°C. For this reason the temperature during development was controlled by immersing solutions in a water bath thermostat at 25.0 ± 0.1°C. Since HCl concentration influences the rate of color development, reliable results will be obtained only if the test solutions of preparations do not contain large amounts of buffering agents.

Comparison of Colors Obtained with A-Substance and N-Acetylglucosamine. Morgan and King (3) have suggested that the color reaction with Ehrlich's reagent is due to an N-acetylglucosamine rest in A-substance. A-substance from hog gastric mucin was found to give colors equivalent to 12–13% N-acetylglucosamine; this estimate

is approximately confirmed. The equivalent *per cent* N-acetylglucosamine of a number of purified A-substance fractions has been found to vary from 10–13%; these values were calculated for colors obtained with 1000 γ of material and for a period of heating with alkali of 15 minutes for A-substance and 5 minutes for N-acetylglucosamine. The fractions were isolated from hog gastric mucin by fractionation with alcohol, sodium sulfate or phenol and alcohol, from pepsin by autolysis or alcohol fractionation, or by treatment of A-substance with papain-HCN or formamide. Morgan and King used results for shorter digestion times of A-substance (6 minutes) for their calculations; their test conditions also differ as has been discussed above. It is of interest that the equivalent *per cent* N-acetylglucosamine accounts for less than half of the 28–30% hexosamine found in A-substance (3, 8, 11).

It should be noted that calculations of equivalent N-acetylglucosamine content are subject to variation depending on the amount of A-substance used for analysis and on the time of digestion of A-substance and N-acetylglucosamine with sodium carbonate solution. A maximum color intensity occurs for most A-substance preparations after digestion at 100°C. with alkali for 15 minutes; a considerably sharper maximum is produced for N-acetylglucosamine after 5 minutes digestion with alkali.

Serological Test (Inhibition of Isoagglutination)

Anti A-(α)-Agglutinin. Pooled serum collected from persons of blood Group B was used. This serum was prepared by the Hyland Laboratories[4] and was selected for reasonable potency before its pooling. All of the serum used by us bore the same lot number. It was dyed with a preservative and stored at 5°C. in the original containers. The undiluted serum just produced incipient agglutination at a dilution of 1:128 when tested against pooled A cells by the method described below. The potency of the serum remained constant during the course of the experiments. The serum was diluted with 24 volumes of 0.9% NaCl solution just before use in the inhibition of isoagglutination studies. Thus, there were present 20 microliters of serum in each tube of a series, while 4 microliters of serum were sufficient to produce just microscopically visible agglutination of the volume of cells used in the inhibition system. This four-fold excess of serum over that required to give incipient agglutination was insufficient, however, to produce complete agglutination of the volume of pooled Group A cells (not selected with reference to subtype) used.

Anti B-(β)-Agglutinin. Pooled serum from persons of blood group A was used. This serum, prepared by Hyland Laboratories, was similar to that containing anti A-(α)-agglutinin. The undiluted serum just produced incipient agglutination at a dilution of 1:256 when tested against pooled B cells by the method described below. As in the case of the anti A-(α)-agglutinin 20 microliters of group A serum were present in each tube of a series, with 2 microliters being sufficient to produce just microscopically perceptible agglutination of the group B cells present. Thus, there was employed a nine-fold excess of serum over that required for incipient agglutination. This amount was almost sufficient to produce complete agglutination of the volume of group B cells used.

Group A Erythrocytes. The cells used in each experiment were pooled from ten individuals, each of Group A. Since the source of the cells was a Red Cross blood bank

[4] Address: 4534 W. Sunset Ave., Los Angeles, California.

center, each batch was obtained from different groups of donors. The unwashed cells were preserved at 5°C. and used until a suspension of them showed microscopically perceptible autoagglutination. They were then discarded. In no case were cells used for longer than 5 days following their collection from the donors. Just before use the cells were washed 4–5 times by suspending them in 4 times their volume of 0.9% NaCl solution, stirring and centrifuging. For use in the inhibition studies a 1% suspension of the freshly washed cells was prepared by diluting an aliquot of the packed cells with 0.9% NaCl solution.

Group B Erythrocytes. The pooled cells from ten individuals of blood group B were treated as described for group A cells.

Experimental Procedure for Determinations of A and of B Activity. The following description applies as well to the procedure for the determination of the inhibition of isoagglutination of group B cells as it does to that for group A cells, with suitable changes in the kind of sera and cells used. It is specifically discussed with reference to A activity only. Solutions of the substances to be tested were prepared in 0.9% NaCl at a concentration of approximately 1 mg./ml. Serial dilutions by two of the test substances with normal saline were made in small culture tubes. In making these dilutions 1 ml. serological pipets were used to mix and then withdraw 0.5 ml. aliquots from each tube for transfer to the next. A fresh pipet was used for each tube in a series. The final volume of test substance solution in each tube was 0.5 ml. Into each tube of the series and into suitable controls 0.5 ml. of 1:25 Group III (B) serum was pipetted. The contents of the tubes were mixed by sharp stroking of the tubes and were then allowed to stand in a water bath at 20–25°C. for one hour. One-half ml. of a 1% suspension of Group II (A) cells in normal saline was then pipetted into each tube of the series. The contents of each tube was again mixed and then allowed to stand for 2 hours at 20–25°C. At the end of this time the tubes were centrifuged for 1 minute in a clinical centrifuge. The examination of the mixture for agglutination was made as follows. The centrifuged cells in each tube in a series were resuspended by stroking the tube, at the same time observing whether there was massive agglutination. In this way it was possible to eliminate certain tubes from further consideration. For the final selection of the first tube in a series which had agglutination the contents were examined under the low-power objective of a microscope. The stage of the microscope was tilted so that when a drop of a cell suspension was streaked across the slide, the cells moved slowly across the field. By observing whether cells which seemed to be aggregated as they entered the field remained so as they moved across it, it was possible to distinguish true agglutination from adventitious clumping. The endpoint of inhibition was taken as the mean of the smallest amount of A substance which entirely prevented clumping of cells (recorded as 0 agglutination) and of that amount which permitted several 2-cell aggregates to be observed in two to three streakings of the suspension across the field (recorded as ± agglutination). In some cases the first tube of a series to show agglutination exhibited it sufficiently strongly that 1–3 2-cell clumps were continually visible in the moving field (recorded as + 1 agglutination). In these latter cases, where there was no tube having ± agglutination, the endpoint was taken as the mean of the amounts of test substance present in the last tube which showed 0 agglutination and in the first tube with + 1 agglutination.

The titer of a preparation, *i.e.*, the microliters of serum neutralized/γ of substance, was calculated by dividing 20 (the number of microliters of serum in the test system)

by the amount of material taken as the endpoint; the amount of serum which reacts with the red cells in the test system is neglected. The experiment described immediately below indicates that the titer of a preparation is essentially independent of the amount of serum used in the test system.

An experiment was performed to investigate the dependence of the inhibition titer of a test substance on the agglutinin concentration. Six sets of tubes were prepared, each set being of serial dilutions by two of an A-substance preparation in normal saline (final volume in each tube; 0.5 ml.). The test substance used in this experiment was material precipitated from hog gastric mucin by 30% Na_2SO_4 and soluble upon electrodialysis of the precipitate.

To each tube within a series, 0.5 ml. of serum, diluted in some cases with normal saline, was added. A different dilution of serum was used for each series. After mixing their contents the tubes were allowed to stand 1 hour in a water bath at 25°C. To each tube 0.5 ml. of a 1% suspension of group A cells in normal saline was added. The tubes were allowed to stand for 2 hours at 37°C. They were then examined for agglutination by the method described above. The results are presented in Table I.

TABLE I

Dependence of Endpoint Upon Amount of Serum Used in Test

Amount of Serum Present in Each Tube (μl)	Amount of Test Substance In Tube Showing Indicated Degree of Agglutination (γ)	
	0	±
500	36.1	18.1
250	18.1	9.0
125	9.0	4.5
62	2.2	1.1
31	2.2	1.1
20	1.1	0.56

Any endpoint obtained by the inhibition test may be in error by a factor of two. This limit of error is fixed partly by the use of serial dilutions by two and partly by the unknown extent to which small differences in experimental conditions affect the phenomenon of agglutination. With these considerations borne in mind the data in Table I can be seen to illustrate the generally linear dependence of inhibition endpoint on agglutinin concentration.

Results

The correlation of the serological activity and the color intensity obtained with Ehrlich's reagent for A-substance fractions prepared by mild procedures from hog gastric mucin[5] is shown in Fig. 1. The preparations were obtained by fractionation with sodium sulfate or

[5] Wilson Laboratories gastric mucin granules, Item No. 443.

phenol-alcohol according to the methods of Morgan and King (3), by alcohol fractionation, by aqueous extraction and by electrodialysis of some of the fractions derived from these procedures. The procedures do not subject A-substance to conditions which are known to degrade it, such as highly acidic or basic solutions at elevated temperatures (3).

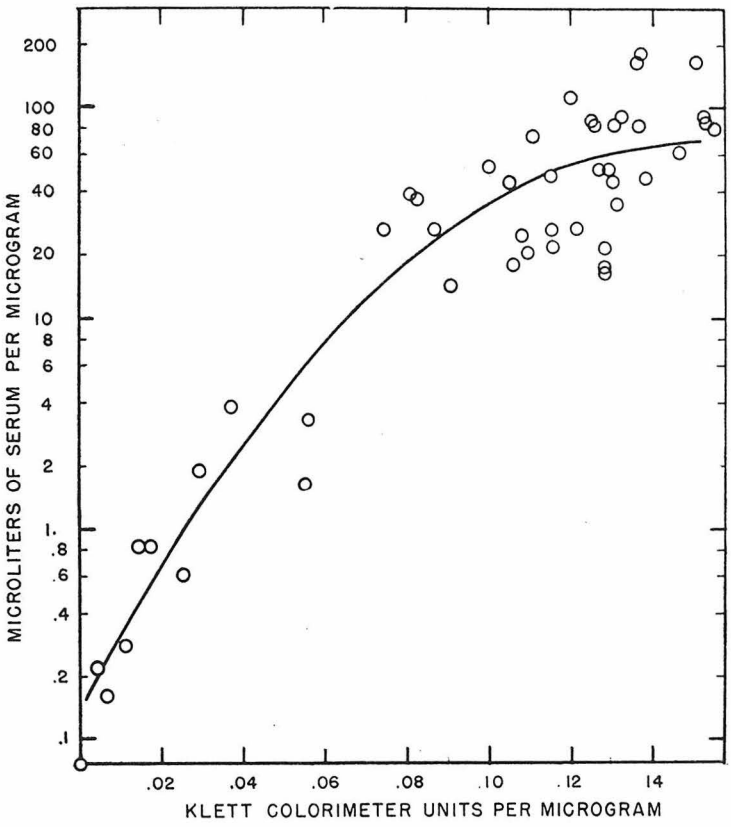


FIG. 1

Relation between Color Intensity with Ehrlich's Reagent and the Isoagglutination Inhibition Titer for A-Substance Preparations from Hog Gastric Mucin

This restriction permits a more accurate estimate of the degree of correlation between the color test and the serological test since degraded materials, which may differ substantially in behavior from undegraded materials in these tests, are omitted from consideration.

Data for 47 separate fractions are plotted in Fig. 1. The serological activity is reported as microliters of serum neutralized/γ of test material. The value of the titer for a preparation was calculated from the mean of the amounts of material found by the serial dilution technique to produce minimal agglutination and complete inhibition of agglutination. Usually only single determinations of titers were made; the precision of the determination is estimated to be $\pm$ 50–100% over the whole range of titers.

The ability of the substance to give a color with Ehrlich's reagent is given on the abscissa of Fig. 1 as Klett Colorimeter units/γ of test material. This value is not entirely independent of the amount of material utilized in the color test, usually about 1000 γ with an over-all range of about 500–1500 γ. However, the values of the slopes (Klett Colorimeter units/γ) at the limits of this range were found to differ by less than 8% from the slope for 1000 γ of material for a number of A-substance preparations. Since these deviations approach the precision of the color test and are small compared to errors in the serological test, it would be expected that the existence of a correlation between the two tests would not be obscured by neglecting these deviations. No corrections for these deviations have been applied to the data in this study. Such corrections would be a desirable refinement as the precision of the methods improve and when the nature of the color reaction is clearly established. The majority of the slopes reported are the mean of two determinations with an average deviation from the mean of 3% for highly active preparations and as much as 10–30% for weakly active preparations (less than 0.5 microliters/γ).

The curve shown in Fig. 1 is the least squares fit of a second-degree parabola to the data; the index of correlation for the curve is 0.95. The data show a normal distribution about the least squares curve; a semi-logarithmic plot of the data is convenient because of the wide range of titers of preparations. Since the precision of the color test is about ten times greater than the serological test (except at very low activities), the least squares solution has not considered the errors which may be involved in the colorimetric estimation. Because of the difficulty of estimating very faint colors at low activities, the estimated titers from the least squares curve below 0.17 microliters/γ are not significant.

The curve in Fig. 1 shows that a significant relation exists between the color with Ehrlich's reagent and blood group A activity. This

TABLE II

*Comparison of Observed and Estimated Titers for Fractions Isolated from Hog Gastric Mucin**

Compound No.	Procedure	Titer (μl serum/γ)		Probability of observed deviation
		Observed	Estimated	
R4-F4	Alcohol fractionation (Landsteiner and Harte)	0.085	0.17	0.27
R5-F1		.29	.20	.56
R4-F1		.30	.27	.87
R5-F2		20	19	.94
R10-F2		8.2	19	.18
R8-F2B		44	27	.43
R4-F2		29	28	.96
R5-F3		111	44	.14
R8-F1		54	54	1.00
R4-F3		63	56	.85
R11-F2		154	59	.13
R8-F2A		42	61	.56
C-51	Procedure of Meyer, Smyth, and Palmer	6.1	2.2	.11
C-49		5.7	8.0	.59
C-54		22	27	.75
C-8		27	35	.68
C-50		21	39	.33
C-4		53	44	.77
C-48		33	65	.28
C-75	Treatment with ammoniacal copper solution	84	45	.32
C-76		84	54	.49
C-77		44	55	.72
R14-F2A	Treatment with alkaline pyridine	48	38	.71
R14-F1A		53	53	1.00

* Notes to Table II.—*R4-F4:* Soluble after addition of 2 vol. EtOH to filtrate of aqueous suspension of mucin. *R5-F1:* Insoluble in aqueous suspension of mucin, pH 4.3, after heating at 90°C. *R4-F1:* Same as R5-F1, duplicate experiment. *R5-F2:* Insoluble on addition of ¾ vol. EtOH to filtrate of aqueous suspension of mucin. *R10-F2:* Obtained by dialysis of R5-F2 for two weeks at 5°C. *R8-F2B:* Residue on electrodialysis of R5-F3 for 5 days. *R4-F2:* Same as R5-F2, duplicate experiment. *R5-F3:* Precipitated between ¾ and 2 vol. EtOH from filtrate of aqueous suspension of mucin. *R8-F1:* Clear supernatant on electrodialysis of R5-F3 for 5 days. *R4-F3:* Same as R5-F3, duplicate experiment. *R11-F2:* Obtained by dialysis of R5-F3 for two weeks at 5°C. *R8-F2A:* aqueous washing of R8-F2B. *C-51:* Precipitated at pH 7 by zinc acetate. Electrodialyzed at pH 5.3, insoluble after electrodialysis. *C-49:* After

TABLE II (*Continued*)

Compound No.	Procedure	Titer (μl serum/γ)		Probability of observed deviation
		Observed	Estimated	
R7-S1B	Treatment with $HCONH_2$ at 150°C. (Landsteiner and Harte)	.51	1.6	.075
R7-F3B		4.3	6.4	.53
R7-S2		11	26	.19
R7-F2A		18	38	.24
R7-F2C		9.7	47	.01
R7-S3		5.4	48	.0006
R7-F2B		11	54	.01
R7-F1		29	64	.21
R7-F4A		7.1	64	.0006
R7-F5A		12	65	.008
R6-F4A	Treatment with papain-HCN (Landsteiner and Harte)	6.9	56	.001
R6-F1A		37	59	.46
R6-F2		48	60	.72
R6-F1B		24	63	.13
R6-F5A		25	65	.13
1A	A-substance preparation by Kabat and Bezer	87	64	.63
M-330	A-substance preparation by Lilly	83	60	.61
960-GM-2	A-substance preparations by Sharp and Dohme	77	57	.64
960-GM-1B		91	55	.43
960-GM-1C		77	59	.67

precipitation by 18 vol. glacial acetic acid, insoluble in water, insoluble after electrodialysis. *C-54:* As for C-51, except soluble after electrodialysis. *C-8:* Obtained after initial precipitation with 3 vol. EtOH, and after precipitation with 18 vol. glacial acetic acid, not dialyzed. *C-50:* As for C-49, except soluble after electrodialysis. *C-4:* Obtained after the initial precipitation with 3 vol. EtOH, but before acetic acid precipitation, not dialyzed. *C-48:* Obtained after precipitation by 18 vol. glacial acetic acid. Material soluble in water and precipitated at 30–35°C. by 30% Na_2SO_4, electrodialyzed. *C-75:* Extracted by phenol from mucin and treated for 1 hr. at room temperature with an ammoniacal copper solution (reagent contained 4.95 g. Cu/l. and was 5.2 *M* in NH_4OH). Adjusted to pH 5 with acetic acid. Dialyzed at room temperature. *C-76:* As for C-75, except treated 6 hr. with the reagent. *C-77:* As for C-75, except treated for 3 hr. with the reagent. *R14-F2A:* A-substance, isolated by Na_2SO_4 fractionation, treated with alkaline pyridine (0.002 *M* NaOH in 90% pyridine) for 2 days and then the material soluble in alkaline pyridine precipitated with acetone, dialyzed. *R14-F1A:* As for R14-F2A except material was insoluble in alkaline pyridine, dialyzed. *R7-S1B:* Soluble on addition of 2 vol. EtOH to formamide solution of

curve can be considered only as an approximation; more data, particularly at low and moderate activities, would be required for a more accurate relationship. Fig. 1 has been used for the estimation of the blood group A activity of, (a) fractions obtained from hog gastric mucin by somewhat more drastic conditions (Table II); (b) A-substances prepared by other investigators from hog stomach or hog gastric mucin (Table II) and (c) fractions obtained from sources other than hog stomach (Table III). Brief descriptions of the treatment and method of isolation of fractions are given in notes to the tables.

Table II shows a comparison of the observed and estimated titers for fractions isolated from hog gastric mucin[6] by alcohol fractionation of a previously heated mucin suspension (8), by treatment with ammoniacal copper solution, alkaline pyridine, formamide (8), papain-HCN (8) or by the procedure of Meyer, Smyth and Palmer (11), which involves a preliminary treatment of mucin with sodium carbonate at 70°C. Comparison of observed and estimated titers are also shown for A-substance isolated by Kabat and Bezer (1) from hog

---

R5-F3. Dialyzed. Soluble in water on dialysis. *R7-F3B:* Soluble on addition of 4 vol. EtOH to solution of R7-F2B. *R7-S2:* Supernatant from first reprecipitation of R7-F1 from 66% EtOH, dialyzed. *R7-F2A:* Insoluble in 90% acetic acid solution of R7-F1. *R7-F2C:* Supernatant from the precipitation of R7-F2B from 90% acetic acid with acetone, dialyzed. *R7-S3:* Supernatant from second reprecipitation of R7-F1 from 66% EtOH, dialyzed. *R7-F2B:* Precipitated on addition of ½ vol. of acetone to supernatant of 90% acetic acid solution of R7-F1, dialyzed. *R7-F1:* Insoluble on addition of 2 vol. EtOH to formamide solution of R5-F3. Reprecipitated twice from 66% EtOH. *R7-F4A:* Insoluble on addition of 4 vol. EtOH to solution of R7-F2B. Reprecipitated from HCl solution with 4 vol. EtOH, dialyzed. *R7-F5A:* Precipitated from 90% acetone solution of R7-F4A. *R6-F4A:* Insoluble on addition of 4 vol. EtOH to solution of R6-F1A. Insoluble in 90% acetic acid solution. *R6-F1A:* Insoluble on addition of 1.5 vol. EtOH to filtered and dialyzed papain-HCN digest (7 days at 37°C.) of R4-F3. *R6-F2:* Supernatant from the precipitation of R6-F1A and R6-F1B with EtOH. *R6-F1B:* Same as R6-F1A except this fraction did not centrifuge readily from alcohol solution and was obtained by Seitz filtration. *R6-F5A:* Insoluble on addition of 4 vol. EtOH to solution of R6-F1A. Soluble in 90% acetic acid solution, precipitated by acetone, and dialyzed. *1A:* Prepared by alcohol fractionation, see reference (1). *M-330:* Prepared from hog stomach linings by peptic autolysis and alcohol fractionation. *960-GM-2:* Prepared from hog gastric mucin by phenol fractionation. *960-GM-1B:* Prepared by autolysis from hog gastric mucin. *960-GM-1C:* Prepared by tryptic digestion and alcohol fractionation from hog gastric mucin.

[6] Except where otherwise indicated, the hog gastric mucin was obtained from Wilson Laboratories (Item No. 443).

TABLE III

*Comparison of Observed and Estimated Titers for Fractions Isolated from Sources other than Hog Gastric Mucin**

Compound No.	Source	Procedure	Titer (μl serum/γ)		Probability of observed deviation
			Observed	Estimated	
C-105	Horse stomach mucosa; (B activity, <.04 μl/γ)		0.21	0.33	0.48
M-336C	Horse stomach prepared by Lilly; (B activity, 4.8 μl/γ)		3.2	9.8	.08
960-P-HS-4	Horse stomach linings prepared by Sharp & Dohme; (B activity, 5.6 μl/γ)		.44	34	.0000
DM	Duodenal mucosa (Wilson)	Untreated source material	.87	.22	.03
C-32	Pepsin (Parke, Davis)		.40	.19	.24
C-33	Pepsin (Wilson)		1.9	.28	.003
C-5	Pepsin	Autolysis procedure of Landsteiner and Harte	2.0	.66	.08
C-22			61	48	.69
C-44	Pepsin	Alcohol fractionation (Landsteiner and Chase)	.049	0	—
C-26			27	52	.30

* Notes to Table III.—*C-105:* From fresh horse stomach mucosa treated according to U.S. Patent 1,829,270 (S. J. Fogelson) and then according to the Na_2SO_4 fractionation method of Morgan and King (3). *M-336C:* Prepared by peptic hydrolysis and alcohol fractionation. *960-P-HS-4:* Prepared by peptic-tryptic digestion and alcohol fractionation. *DM:* Wilson Laboratories duodenal mucosa preparation. *C-32:* Parke, Davis and Co. pepsin (1:3000). *C-33:* Wilson Laboratories pepsin (1:10,000), Item No. 414. *C-5:* From Wilson pepsin (see C-33), autolyzed, precipitated by 66% EtOH and then dissolved. Finally precipitated by 50% EtOH. *C-22:* From Wilson pepsin (see C-33), autolyzed, precipitated by 62% EtOH and then by 9 vol. glacial acetic acid and 5 vol. acetone; dialyzed and precipitated by 4 vol. EtOH plus sodium acetate. *C-44:* From Wilson pepsin (See C-33), precipitated by acetic acid from aqueous solution, washed. *C-26:* From Wilson pepsin (see C-33), obtained

TABLE III (*Continued*)

Compound No.	Source	Procedure	Titer (μl serum/γ)		Probability of observed deviation
			Observed	Estimated	
C-27	Blood group A stromata	Procedure of Kossjakow	2.4	6.8	.10
R1-F1	Blood group A stromata	Procedure of Hallauer	<.008	0	—
R1-F3			<.008	0	—
C-45			.18	0	—
R1-F5			<.14	.18	—
R1-F2			.35	.19	.34
R1-F4			.067	.20	.08
C-30			.067	.26	.03
C-28			.18	.28	.49
C-73			.77	.56	.61
C-34	Blood group A erythrocytes	Procedure of Hallauer	.011	0	—
C-2			<.09	0	—
C-46			.13	0	—
C-57			<.003	0	—
C-3			<.09	.18	—
C-56			.92	.21	.02
C-1			.083	.24	.09
C-113	Pseudo-mucinous ovarian cysts (blood group A)	Alcohol fractionation	.42	0	—
C-119			1.8	2.5	.60

by two 65% alcohol precipitations at pH 8.5, dialyzed. *C-27:* Treated according to Kossjakow's procedure as far as one alcohol precipitation. *R1-F1:* Extract with 95% EtOH. *R1-F3:* First 25% EtOH extract. *C-45:* Boiling absolute alcohol extract. *R1-F5:* Extract with 10% EtOH. *R1-F2:* Extract with 50% EtOH. *R1-F4:* Second 25% EtOH extract. *C-30:* First 25% EtOH extract. One acetone precipitation. *C-28:* Second 25% EtOH extract. One acetone precipitation. *C-73:* Aqueous extract. Precipitated once with acetone. *C-34:* Extract with 10% EtOH. One acetone precipitation. *C-2:* First 25% EtOH extract. One acetone precipitation. *C-46:* Boiling absolute alcohol extract. *C-57:* Material precipitated by 66% EtOH from aqueous extract of material extracted by 50% EtOH according to Hallauer's procedure. *C-3:* Second 25% EtOH extract. One acetone precipitation. *C-56:* Material precipitated by 50% EtOH from aqueous extract of material extracted by 50% EtOH according to Hallauer's procedure. *C-1:* Extract with 50% EtOH. One acetone precipitation. *C-113:* Material precipitated by 50% EtOH from the native fluid of a pseudomucinous

gastric mucin and for A-substances isolated from hog stomach and hog gastric mucin by the Sharpe and Dohme and Lilly laboratories.[7]

Column 5 in Table II gives the calculated probability that the deviations between observed and estimated titers are due to a normal distribution of error. Probabilities less than 0.05 indicate deviations from the estimated titers which are significantly larger than deviations from the least squares curve shown in Fig. 1. The agreement between observed and estimated titers is satisfactory except for some of the fractions isolated by formamide and papain procedures. However, these two procedures are known to cause extensive degradation of A-substance (3, 8) and to decrease the isoagglutination titer of preparations. In these cases it appears that the degradation has disturbed the structure of A-substance to such a degree that the serological specificity is lost while the functional groups responsible for the color test are relatively unaffected. The other procedures of isolation apparently effect no substantial degradation of A-substance detectable by means of a comparison of the observed and estimated titers.

Table III summarizes the results of observed and estimated blood group A titers for fractions isolated from pepsin (8, 9), blood-group A erythrocytes and stromata (12, 13), horse stomach, and pseudomucinous ovarian cyst fluids of individuals of blood group A.[8] Materials isolated from horse stomach mucosa are often known to possess blood group B activity; titers against B cells are shown parenthetically in Table III for these preparations. In several cases unpurified materials were analyzed by the serological and color tests; comparisons of these results would not be expected to be as satisfactory as in those cases where some means has been resorted to for the separation of inactive materials which may interfere with the tests.

Probabilities are not calculated for those cases in which the estimated titer is below the limit of the colorimetric method, 0.17 microliters/γ, or in those cases where the observed titer is only known to be less than

---

ovarian cyst removed from an individual of blood group A. Material dialyzed. *C-119:* Material soluble in 50% EtOH but insoluble in 65% EtOH from same cyst fluid as C-113. Material reprecipitated once and dialyzed.

[7] We are indebted to Dr. E. Brand for a sample of Kabat's preparation, and to Dr. R. H. Barnes of Sharpe and Dohme, Inc., and Dr. J. A. Leighty of Lilly Research Laboratories for other samples of A-substance and AB mixture.

[8] We are indebted to Dr. R. W. Hammack of the Hospital of the Good Samaritan, Los Angeles, California, for providing us with samples of fluids removed from ovarian cysts.

a given value. These results are of interest since they indicate that the colorimetric method is usually in qualitative agreement with the serological test for fractions of no, or very low, activity.

The results for material isolated from horse stomach are pertinent to the question of the behavior of B-substance isolated from this source in the color test. Recently Morgan and Waddell (14) have observed that B and O substances isolated from pseudo-mucinous ovarian cyst fluids give color tests after treatment with alkali. The agreement between the observed and estimated blood group A titers for the horse stomach preparations shown in Table III is satisfactory either when the B activity of the substance is low (Compound C-105) or when the serological test shows appreciable A activity (Compound M-336C). When a compound shows appreciable B activity but little A activity (Compound 960-P-HS-4), the color test still predicts a very high A activity, the agreement in this case being very poor. This evidence would appear to suggest that B-substance or some material associated with it in horse stomach also gives the color test or that the A-substance factor has been almost completely degraded by the peptic-tryptic digestion used in the preparation of this compound.

The deviations between observed and estimated titers for the remainder of the results in Table III have calculated probabilities which are usually greater than the value of 0.05 of the standard of significance. Some of the probabilities fall somewhat below 0.05 for preparations of low activities; this might be expected, as errors in the colorimetric procedure, which were not considered in the least squares solution, become appreciable at low color intensities.

Discussion

The results which have been presented indicate that the functional groups responsible for the color reaction with Ehrlich's reagent are an inherent feature of the structure of A-substance. The groups appear to be common to the materials possessing A activity derived from human erythrocytes and pseudo-mucinous ovarian cyst fluids as well as from hog gastric mucin, pepsin and horse stomachs. It would not be expected that detailed differences in the A-substances isolated from these various sources could be revealed in this study due to the lack of precision in the correlation of the serological test and the color test.

Preliminary evidence from materials isolated from horse stomach would appear to indicate that B substance isolated from this source

also possesses similar functional groups; further investigation is required to establish these observations. It is evident that a knowledge of the specificity of the color reaction is important as a part of the general problem of interpreting serological specificity in terms of the chemical structure of the blood group substances.

While the colorimetric procedure has proved to be a useful supplement to serological tests in our work, it may be helpful to point out several difficulties in applying the procedure in its present form as a general method for the estimation of isoagglutination titers of A-substance preparations. First, the method requires a preliminary calibration with the aid of the relatively inaccurate serological test in case one is interested in interpreting the results in terms of serological titers. The empirical relation which is derived in this way will probably be difficult to obtain in a more direct manner until further investigation establishes the chemical structure of A-substance. Another feature restricting the usefulness of the colorimetric method is the fact that the estimated isoagglutination titers may be considerably in error if A-substance has been degraded significantly. This limitation imposes the necessity of determining by preliminary investigation whether the colorimetric method is suitable for the prediction of isoagglutination titers of fractions obtained by various procedures.

Finally, certain limitations in the color test restrict a routine application of the method. For example, solutions of preparations must be essentially neutral and contain no significant amount of buffer since the adjustment of the pH during the sodium carbonate digestion of A-substance as well as on addition of Ehrlich's reagent is critical. Occasionally preparations give turbidities in the final solutions and thus prevent accurate measurements of color intensity. Difficulties of this kind could probably be resolved by altering the experimental conditions of the color test.

Summary

The intensity of the color produced by the reaction of A-substance with Ehrlich's *p*-dimethylaminobenzaldehyde reagent after preliminary treatment of A-substance preparations with alkali has been correlated with the isoagglutination inhibition activity of the original preparations. The use of this correlation to estimate the potency of A-substance preparations has been described. The color reaction with Ehrlich's reagent has been observed for blood group A specific preparations

isolated from hog gastric mucin, pepsin, blood group A erythrocytes and stromata, and human ovarian cyst fluids, and for blood group AB specific preparations from horse stomachs.

References

1. Kabat, E. A., and Bezer, A. E., *J. Exptl. Med.* **82**, 207 (1945).
2. Morgan, W. T. J., and Elson, L. A., *Biochem. J.* **28**, 988 (1934).
3. Morgan, W. T. J., and King, H. K., *Biochem. J.* **37**, 640 (1943).
4. King, H. K., and Morgan, W. T. J., *Biochem. J.*, **38**, *Proc.* x (1944).
5. Jorpes, E., and Thaning, T., *J. Immunol.* **51**, 221 (1945).
6. Holm, G. E., and Greenbank, G. R., *J. Am. Chem. Soc.* **45**, 1788 (1923).
7. Breuer, R., *Ber.* **31**, 2198 (1898).
8. Landsteiner, K., and Harte, R. A., *J. Exptl. Med.* **71**, 551 (1940).
9. Landsteiner, K., and Chase, N. W., *J. Exptl. Med.* **63**, 813 (1936).
10. Adams, R., and Coleman, C. H., *Organic Syntheses* **2**, 17 (1922).
11. Meyer, K., Smyth, E. M., and Palmer, J. W., *J. Biol. Chem.* **119**, 73 (1937).
12. Kossjakow, P. N., *Z. Immunitäts.* **99**, 221 (1941).
13. Hallauer, C., *Z. Immunitäts.* **83**, 113 (1934).
14. Morgan, W. T. J., and Waddell, M. B. R., *Brit. J. Exptl. Path.* **26**, 387 (1945).

A Study of the Isolation of Blood Group A-Specific Substance from Commercial Hog Gastric Mucin and Some Observations on the Separation of A-Substance from Other Natural Sources[1]

David H. Brown, Edward L. Bennett, George Holzman and Carl Niemann

The serological properties of blood group A-specific substances[2] isolated from a variety of sources (for bibliography, see (1)) are the most characteristic features of these preparations, and, consequently, immunological techniques have been relied upon to evaluate starting materials and preparative procedures. However, the data of previous investigators have permitted only limited comparisons to be made of the efficacy of different procedures for isolating A-substance (2, 3). This situation, which has been commented upon before (3), exists because of the variation in the methods used by different authors in

---

[1] Contribution No. 1085, Gates and Crellin Laboratories of Chemistry, California Institute of Technology. Published in Arch. Biochem. 13 421 (1947). This work was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology

[2] By "group A-specific substance" here, is meant any material effective in inhibiting isoagglutination of human A erythrocytes by type B serum and also effective in inhibiting lysis of sheep erythrocytes by human A-cell immune rabbit sera. Evidence has recently been presented (23) that two different group-specific substances are obtainable from human urine of type A individuals, and that one of these materials is effective in inhibiting hemolysis of sheep erythrocytes by immune rabbit sera while the other is active in inhibiting isoagglutination of human A cells. We have no evidence for the existence of two such different group-specific substances. We have not examined urine, however, as a source of group-specific materials.

carrying out the serological tests and the unpredictable degree to which such tests are influenced by changes in experimental details. Hence, we believe that questions involving the usefulness of various natural products as starting materials for the preparation of A-substance and, particularly, of the most advantageous procedure for effecting its isolation can best be answered by the isolation of a large number of A-substance preparations and by the parallel testing of these preparations by several serological procedures. Accordingly, we have undertaken a comparative study of the preparative procedures described in the literature (for references, see Table I). Most of our work has been done with commercial hog gastric mucin, a material in which A-substance has long been known to be abundantly present. By these experiments we have been able to secure definite evidence bearing on the question of the similarity of A-substance preparations isolable in different ways from hog mucin, and it has been possible to determine which of the several preparative methods that have been proposed is the most satisfactory from the standpoint of yield and potency of product. It has also been possible to compare A-substance preparations obtained from other sources with those obtained from hog gastric mucin.

Experimental

Serological and other analytical procedures. Two serological tests have been used. The inhibition of hemolysis test, which will be discussed in detail shortly (8), makes use of a colorimetric determination of the extent of lysis of sheep erythrocytes by human A-cell immune rabbit sera in the presence of guinea pig serum as a source of complement. The

precision of the hemolysis test is high, and the results obtained by it can be used to distinguish preparations which differ by only 10-20% in serological activity. Each of the fractions obtained has also been assessed by the inhibition of isoagglutination test, using the same lot of human type B serum throughout, and using pooled human type A erythrocytes. In this latter test preparations must differ in activity by approximately 100% to be distinguishable. A detailed description and discussion of the inhibition of isoagglutination test has been given (9). We have also measured the amount of color given by each fraction when treated, after alkaline digestion, with a modified Ehrlich reagent (p-dimethylaminobenzaldehyde) (9). The intensity of color obtained with this reagent after heating blood group A-specific substance with alkali has been found to be correlated with the isoagglutination inhibition titers of the specific substance preparations (9). Since the existence of the correlation is dependent in part upon differences in preparations detectable by means of the inhibition isoagglutination test, only very little correlation is demonstrable for the relatively similar and potent preparations discussed below. However, it has been found that for these preparations there exists a marked correlation between the results of the color test and of the relatively precise inhibition of hemolysis test. For this reason the color produced with Ehrlich's reagent is interpreted as a characteristic property of blood group A-specific substance.

The separation of A-substance from hog gastric mucin. The general isolation procedures employed were: 1, sodium sulfate fractionation from aqueous solution (2); 2, alcohol fractionation from aqueous solution (3); 3, alcohol fractionation from a 90% phenol solution (2); 4, electrodialysis followed by alcohol fractionation; 5, a combination of two or

more of the foregoing procedures. Table I contains abbreviated descriptions of the method of isolation of each fraction that is considered in this communication.

The separation of A-substance from human erythrocytes. The alcohol extraction procedures of Hallauer (7) and of Kossjakow (10) have been followed, with large amounts of dried cells and with dried stromata obtained by centrifuging aqueous suspension of cells from which hemoglobin had been removed by repeated washing. Material extracted by boiling absolute ethanol from dry stromata (7), fraction 45 (see Table I), as well as fractions arising in a similar way from whole erythrocytes or arising from either source by extraction with 95% ethanol in the cold, has nearly as much potency in inhibiting isoagglutination and in inhibiting hemolysis as any of the other fractions arising from the alcohol extraction procedure of Hallauer (7). This ethanol-soluble material is obtained as a major part of all material extracted from dry stromata (7% yield) and was discarded by Hallauer. Because of the insolubility of the material in water, suspensions of it were prepared for serological testing by dilution of an absolute alcohol solution with physiological salt solution. The activity of fraction 45 in inhibiting isoagglutination and in inhibiting hemolysis is about 500 times less than that of the highly active preparation, fraction 66 (see Table I), from hog gastric mucin. Several other materials (see Table I) have been obtained by alcohol extraction in 0.2-0.3% yield from lyophilized erythrocytes and these fractions have all had from 150 to 300 times less activity in the inhibition of isoagglutination test than a potent preparation from hog mucin. Digestion of stromata with crude pancreatin has given a fraction, 161, obtained in 10% yield, which has the same order of activity as fractions obtained by alcohol extraction from erythrocytes.

The extremely low yield and serological activity of group A-specific substance isolated from human erythrocytes is illustrated by the data in Table I (cf. also Bray, Henry and Stacey (11)). This situation may be due to the relatively minute concentration of active material in erythrocytes or to the possibility that the procedures we have used in extracting it are relatively ineffective. This latter possibility is suggested by recent experiments of Calvin and coworkers (12) which are in agreement with earlier observations of Schiff and Adelsberger (13) and Landsteiner and van der Scheer (14). It is probable that our alcohol-soluble fraction 45 obtained as a major fraction from stromata is similar to these lipid-rich fractions obtained by other workers. The similarity in yield of preparations with comparable activity from stromata and from whole erythrocytes is evidence that a large part of the active material was retained in the course of lysing the erythrocytes and washing the insoluble residue. This is in contrast with what might be expected from the results reported by Calvin et al. (loc. cit.). Further evidence is given by the experiments of Belkin and Wiener (15) who found that the titer of A-substance in their stromata preparations was higher than that in the parent erythrocytes and that the ratio of these titers was inversely proportional to the yield of stromata obtained. Thus, the unsatisfactory yield of A-substance from human erythrocytes is most probably attributable not to the inefficacy of the isolation procedures but rather to the very small amount of active material present.

The separation of A-substance from human ovarian cyst fluids. According to the observations of Yosida (16) and the extensive investigations carried

out by Morgan and Van Heyningen (17), King and Morgan (18), and Morgan and Waddell (19), the fluid from pseudomucinous ovarian cysts may contain a blood group-specific substance in water-soluble form which has similar serological specificity to that present in the stromata of the individual's erythrocytes. We have examined such cyst fluids, and from one containing A-specific substances (Fluid No. 162, Table I), have separated two preparations by alcohol fractionation. One of these, fraction 113, precipitated by 50% (v/v) ethanol, has about one-third the potency of the native cyst fluid in inhibiting hemolysis; the other, fraction 119, precipitated by 66% (v/v) ethanol, has about twice the potency. It may be significant that fraction 119 has as much antihemolytic activity as some of the less pure fractions, 109 and 115 (see Table I), from hog gastric mucin, and yet has 4-6 times less potency in inhibiting isoagglutination than these fractions. Morgan and Van Heyningen reported (17) no such discrepancy in the activities of two materials isolated from cyst fluids by rejection of material soluble in 90% phenol.

The specific substance in commercial hog pepsin. We have isolated from Wilson pepsin (1:10,000, soluble powder), in the small yield of 1-2%, fractions 22 and 26 which are extremely potent in inhibiting hemolysis, one of which, fraction 22 (see Table I), also has a high degree of activity in inhibiting isoagglutination. These materials are more active as antihemolytic factors than any other substance we have obtained directly from any source. Only fractions 47 and R.7 F.5A, obtained from hog gastric mucin by other than the usual mild procedures (see Table I and the discussion below), have activities nearly or equally as high. Fractions 22 and 26 do not exhibit the augmented equivalent N-acetylglucosamine content of the somewhat degraded preparations 47, 48, R.6 F.5A, and R.7 F.5A and give somewhat less color in the modified Ehrlich procedure (9) than our highly active material from mucin, fraction 66. In the case of fraction 22, obtained according to Landsteiner and Chase (6) by alcohol fractionation of an aqueous solution of pepsin previously heated at pH 6 and pH 3.5, there is no indication of extensive degradation, judged from its still relatively high inhibition of isoagglutination titer. This titer of fraction 26, however, is not commensurate with its antihemolytic activity and, thus, the autolysis procedure of Landsteiner and Harte (3) must be regarded as bringing about some degradation of the specific substance. It appears, then, that the specific substance isolable from pepsin (fractions 22 and 26) differs from that obtained from hog gastric mucin (fraction 66), 1, in having a lower equivalent per cent N-acetylglucosamine, 2, in having a somewhat diminished inhibition of isoagglutination potency, and 3, in having enhanced activity in inhibiting hemolysis. The extent to which these properties are related is unknown. When the serological properties of the fractions from pepsin are considered together with their behavior in the modified Ehrlich procedure, it is apparent that these specific substances partially resemble the somewhat degraded preparations, 47 and R.7 F.5A, whose preparations are discussed below. A preparation of Parke, Davis and Co. pepsin (1:3,000, granulated) proved to be valueless as a source of A-substance, confirming the findings of Freudenberg et al. (20) that different preparations of pepsin vary widely in their content of group A-specific substance. The Fairchild Bros. and Foster, 1:15,000, pepsin used by Landsteiner and co-workers in their isolation studies (3, 6) would appear to have the highest concentration of A-substance of any commercial preparation.

Discussion

The data in Table I indicate that, irrespective of the method of isolation used, no fraction was obtained from hog gastric mucin which had more than 2-4 times the activity in the inhibition of isoagglutination test or 2-3 times the activity in the inhibition of hemolysis test of a centrifuged (Sharples), undialyzed suspension of the original mucin (fractions 62 and 135, Table I). The inhibition of hemolysis potency of the most active fraction derived by application of any one isolation procedure alone was the same, within 20%, as the potency of one obtained by any other procedure (cf. fractions R.1 F.2A, R.2 F.2A, R.3 F.2, R.5 F.3, 87, 128, 143, 120, 31, 66, 69, Table I), which might be taken as evidence that the active material obtained is a fairly well-defined preparation, although not necessarily homogeneous. It is also apparent from the data of Table I that, within this group of similar fractions, significant differences occur with respect to the equivalent N-acetylglucosamine content[3] (9), and even with respect to the serological activity itself, indicating that preparations of A-substance (e.g., fractions 66, 68, 87, R.3 F.2) which are more active than those obtainable by the use of the hitherto described procedures (2, 3) can be isolated by relatively complex, yet still mild, techniques. These more active, undegraded preparations have perhaps a 20% higher A-substance content than those (fractions R.5 F.3, 128, 143, 31)

---

[3] Various values can be found for the equivalent N-acetylglucosamine content of an A-substance preparation depending upon the experimental conditions selected for the analysis of N-acetylglucosamine itself and for that of the specific substance preparations. However, the values determined for the different A-substance preparations under standardized conditions are highly significant in a relative rather than an absolute way, since the existence of correlations with the serological tests can be demonstrated.

Table I

Properties of Some Blood Group A-Specific Substance Preparations

Starting material	Procedure followed in isolation	Note	Fraction number	Yield (from starting material)	Inhibition of hemolysis titer[a,bbb]	Inhibition of isoagglutination titer[b,bbb]	Color with modified Ehrlich reagent[c,bbb]
				percent			
Hog gastric mucin granules, Wilson Lab. Item No. 443	Sodium sulfate fractionation of a centrifuged aqueous suspension (2).	d	R.1 F.2A	10	0.075±0.005	45±15	11.7±0.3
			R.2 F.2A	15	0.11 ±0.01	45±15	12.1±0.3
	Electrodialysis of uncentrifuged aqueous suspension followed by sodium sulfate fractionation	e	R.3 F.2		0.090±0.010	60±20	12.1±0.3
	Aqueous suspension centrifuged once in Sharples at pH 4.8. Electrodialyzed. Fractionated with ethanol.	f	62	75	0.23 ±0.01	40±10	7.1±0.4
		g	111	33	0.19 ±0.01	75±30	8.4±0.3
		h	112	26	0.24 ±0.00	50±15	7.5±0.3
		i	87	11	0.093±0.005	95±30	11.8±0.3
	Aqueous suspension fractionated with ethanol after preliminary heating at pH 4.2 (3).	j	R.4 F.3	25	0.12 ±0.01	80±20	11.0±0.3
			R.5 F.3	29	0.11 ±0.01	85±20	11.0±0.2
	Aqueous suspension fractionated with ethanol after centrifuging twice at pH 4.4 in Sharples.	k	125	60	0.18 ±0.01	45±15	9.0±0.2
		l	123	34	0.24 ±0.01	40±15	8.5±0.3
		m	128	21	0.11 ±0.00	75±20	11.8±0.1
		n	135	72	0.18 ± 0.02	45±15	7.6±0.2
		o	142	38	0.24 ± 0.00	45±15	8.1±0.2
		p	143	22	0.11 ± 0.01	85±20	10.6±0.1
	Aqueous suspension fractionated with ethanol after centrifuging at pH 4.4 and then at pH 7.2 in Sharples.	q	97	65	0.18 ± 0.01	45±15	9.0±0.1
			124	51	0.17 ± 0.01	40±15	9.3±0.2

Table I -Continued

Starting material	Procedure followed in isolation	Note	Fraction number	Yield (from starting material)	Inhibition of hemolysis titer[a,bbb]	Inhibition of isoagglutination titer[b,bbb]	Color with modified Ehrlich reagent[c,bbb]
				per cent			
Hog gastric mucin granules, Wilson Lab. Item No. 443	Aqueous suspension fractionated with ethanol after centrifuging at pH 4.4 and then at pH 7.2 in Sharples.	r	109	30	0.25 ±0.01	40±10	11.0±0.2
			115	33	0.24 ±0.01	45±15	11.1±0.1
		s	120	15	0.10 ±0.00	75±20	9.3±0.1
			121	17	0.10 ±0.00	80±20	9.6±0.2
		t	127	53	0.17 ±0.00	45±15	10.6±0.1
			129	51	0.18 ±0.00	45±15	10.6
	Ethyl alcohol fractionation of a centrifuged 90% phenol solution (2).	u	39	49	0.20 ±0.04	40±15	11.2±0.2
		v	52	32	0.13 ±0.01	50±15	11.4±0.1
		w	31	13	0.11 ±0.00	55±10	11.4±0.1
		x	36	10	v. Note uu	55±15[uu]	9.5±0.5[uu]
	Ethyl alcohol fractionation of aqueous solution of fraction 52.	y	66	4	0.080±0.010	110±30	12.7±0.1
		z	68	5	0.11 ±0.00	90±30	12.7±0.1
	Sodium sulfate fractionation of aqueous solution of fraction 52.	aa	69	1	0.10 ±0.01	75±20	10.7±0.1
		bb	71	22	0.12 ±0.01	80±20	12.7±0.1
Hog Gastric Mucin(4)[4,5]	Ethyl alcohol fractionation of 90% phenol solution (2,4).[4]	cc	Kabat 1A(4)		0.10 ±0.01	50±20	11.3±0.1
			960-GM-2[4]		0.14 ±0.01	55±25	10.0±0.2
	Tryptic digestion followed by alcohol fractionation	dd	960-GM-1B[4]		0.17 ±0.02	55±25	9.6±0.2
			960-GM-1C[5]		0.13 ±0.01	55±25	10.5±0.1
Hog Stomach Linings	Autolysis, followed by alcohol fractionation.[6]	ee	M.330[6]		0.14 ±0.01	55±25	10.7±0.1

Table I - Continued

Starting material	Procedure followed in isolation	Note	Fraction number	Yield (from starting material)	Inhibition of hemolysis titer[a,bbb]	Inhibition of isoagglutination titer[b,bbb]	Color with modified Ehrlich reagent[c,bbb]
				per cent			
Hog gastric mucin granules Wilson Lab. Item No. 443	Separation of "neutral polysaccharide" by selective adsorption and precipitation (5).	ff	47	2	0.065±0.010	30±10	13.3±0.5
	Fractionation of part of the product by precipitation with sodium sulfate.	gg	48	5	0.080±0.020	30±10	12.3±0.1
	Digestion of fraction R.4 F.3 with papain-HCN. Subsequent fractionation with ethanol and with acetic acid and acetone (3).	hh	R.6 F.5A	31[vv]	0.10±0.01	25±10	12.3±0.2
	Heating fraction R.5 F.3 with formamide. Subsequent fractionation with ethanol, acetic acid and acetone (3).	ii	R.7 F.5A	26[ww]	0.050±0.005	10± 5	13.4±0.3
Pepsin, soluble powder, 1:10000, Wilson Lab.	Alcohol fractionation of an aqueous solution previously heated at pH 6 and pH 3.5 (6).	jj	22	1	0.050±0.005	70±15	11.6±0.3
	Autolysis, followed by alcohol fractionation (3).	kk	26	2	0.040±0.005	35±10	11.7±0.1
Fluid from pseudomucinous ovarian cyst from patient of blood group A.[7]	Centrifugation, followed by alcohol fractionation.	ll	162		0.65 ±0.20	4.5±1.5	1.7±0.3
		mm	113		1.9 ±0.3	4 ± 3	0.3±0.2
		nn	119		0.27 ±0.03	7 ± 5	3.4±0.3

Table I - Continued

Starting material	Procedure followed in isolation	Note	Fraction number	Yield (from starting material)	Inhibiton of hemolysis titer[a,bbb]	Inhibition of isoagglutination titer[b,bbb]	Color with modified Ehrlich reagent[c,bbb]
				per cent			
Lyophilized erythrocytes, pooled from a large number of donors of blood group A.[8]	Successive extractions with ethanol-water solutions of decreasing alcohol content, followed by concentration of extract and precipitation with acetone (7). Subsequent fractionation with ethanol.	oo	1	0.3	16±2	0.1±0.04	less than 0.3
		pp	56	7[xx]	13±2	0.9±0.5	"
		qq	57	22[xx]	140±15	less than 0.03	"
		rr	169	60[xx]	45±5		"
Lyophilized stromata from pooled erythrocytes from donors of blood group A[yy].	Extraction with boiling absolute ethanol (7).	ss	45	7 0.2[zz]	34±3	0.2±0.06[aaa]	less than 0.3
	Digestion with crude pancreatin at pH 7.9 at 40°C.	tt	161	10 0.3[zz]	29±3		0.5±0.3

[4] We are indebted to Dr. E. Brand for samples of Kabat's preparation, 1A, and for two Sharp and Dohme preparations, 960-GM-1B and 960-GM-2, as well as information concerning the methods of preparation of the latter two materials.

[5] We are indebted to Dr. R.H. Barnes, of Sharpe and Dohme, Inc., for a sample of preparation 960-GM-1C and for information concerning the method used in its preparation.

[6] We are indebted to Dr. J.A. Leighty, of the Lilly Research Laboratories, for a sample of preparation M-330 and for information concerning the method used in its preparation.

[7] We are indebted to Dr. Roy W. Hammack, of Pathology Laboratories of the Hospital of the Good Samaritan, Los Angeles, Cal., for a sample of fluid aspirated from a pseudomucinous ovarian cyst (No. B-3731-45) removed from a patient of blood group A.

Notes to Table I: [8] We are indebted to the Hyland Laboratories, Los Angeles, Cal., for a sample of lyophilized, pooled erythrocytes removed from human donors of blood group A. The erythrocytes were washed twice with physiological saline prior to lyophilization.

a. γ of test substance present in system in which erythrocytes are 50% hemolyzed. b. μl. of serum completely inhibited/γ of test substance. c. Expressed as equivalent per cent of N-acetylglucosamine in test substance. d. Insoluble in 30% sodium sulfate. Precipitate electrodialyzed. Material from supernatant. e. Insoluble when supernatant after electrodialysis made 30% in sodium sulfate. Dialyzed. f. Material from undialyzed centrifugate. g. Material from supernatant of electrodialyzed centrifugate. h. Material precipitated upon electrodialysis of centrifugate. i. Material soluble when supernatant after electrodialysis made 47% (v/v) in ethanol but insoluble when made 65% (v/v) in ethanol. Again electrodialyzed. Material from supernatant. j. Material soluble in 40% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Not dialyzed. k. Material from dialyzed centrifugate. l. Material insoluble in 40% (v/v) ethanol. Dialyzed. m. Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Dialyzed. n. Material from undialyzed centrifugate. o. Material insoluble in 40% (v/v) ethanol. Not dialyzed. p. Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Not dialyzed. q. Material from dialyzed centrifugate. r. Centrifugate. Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Upon reprecipitation, insoluble in 45% (v/v) ethanol. Dialyzed. s. Centrifugate. Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Upon reprecipitation, soluble in 45% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Dialyzed. t. Centrifugate. All of material insoluble in 65% (v/v) ethanol. Dialyzed. u. Material insoluble in 10% (v/v) ethanol. Not dialyzed. v. Material insoluble in 10% (v/v) ethanol. Dialyzed. w. Material from supernatant after electrodialysis of fraction 39. x. Material precipitated by electrodialysis of fraction 39. y. Material soluble in 47% (v/v) ethanol but insoluble in 65% (v/v) ethanol. Electrodialyzed. Material from supernatant. z. As in y. except material precipitated upon electrosis. aa. Precipitated by 30% sodium sulfate. Electrodialyzed. Material from supernatant. bb. As in aa, except material precipitated upon electrodialysis. cc. Preparation made by Sharpe and Dohme. dd. Preparation made by Sharpe and Dohme. ee. Preparation made by Lilly Research Laboratories. ff. Material not precipitated by $Zn(OH)_2$ at pH 7.1 but precipitated by acetone-acetic acid. Material from supernatant after electrodialysis of final precipitate. gg. Precipitated by basic lead acetate, then by 75% (v/v) ethanol, then by 94% (v/v) acetic acid. Not subjected to precipitation by $Zn(OH)_2$. Instead, extracted by water. Material insoluble when extract made 30% in sodium sulfate. Electrodialyzed. hh. Insoluble in 65% (v/v) ethanol after digestion and dialysis. Then insoluble in 75% (v/v) ethanol containing HCl. Soluble in 90% (v/v) acetic acid, but insoluble upon addition of acetone. Dialyzed. ii. Insoluble in 65% (v/v) ethanol in formamide. Precipitated twice by 68% (v/v) ethanol from water and precipitated from 50% acetone-40% acetic acid. Precipitated by 80% (v/v) ethanol from HCl solution. Dialyzed. Precipitated by 90% acetone. Dialyzed. jj. Material insoluble in 65% (v/v) ethanol. Reprecipitated. Dialyzed. kk. Precipitated by 63% (v/v) ethanol twice, then by acetone-acetic acid, then by ethanol. ll. Total solids from the centrifuged fluid. mm. Material insoluble when fluid made 1% in sodium acetate

and 50% (v/v) in ethanol. Precipitate dialyzed and the non-dialyzable suspension filtered. Filtrate lyophilized. nn. Material soluble when fluid made 50% (v/v) in ethanol but insoluble at 66% (v/v) ethanol. Precipitate taken up in water, filtered through Seitz pad and reprecipitated by 66% (v/v) ethanol. Precipitate taken up in water, filtered, filtrate dialyzed. Solution filtered through Seitz pad. Heavily opalescent filtrate lyophilzed. oo. Material insoluble in boiling absolute ethanol extract but soluble in an approximately 50% ethanol extract. Extract concentrated at room temperature, filtered through a Seitz pad. Fraction 1 precipitated by 5 volumes of acetone from a solution 0.9% in NaCl. Not dialyzed. pp. Material (from fraction 1) which is insoluble in absolute ether and in absolute ethanol under reflux but which is soluble in water at room temperature. Fraction 56 precipitated when aqueous extract made 50% (v/v) in ethanol. Not dialyzed. qq. Like fraction 56 except material soluble when extract made 50% (v/v) in ethanol, but insoluble at 66% (v/v) ethanol. Not dialyzed. rr. Like fraction 57 except material soluble when extract made 66% (v/v) ethanol. Not dialyzed. ss. Material extracted by boiling absolute ethanol. Extract concentrated under reduced pressure at 35°C. and the residue lyophilized. tt. Material soluble and non-dialyzable after digestion for 42 hours and inactivation at 85°C. for approximately 30 minutes. uu. Analytical data in doubt because of slight solubility of the fraction in physiological salt solution. vv. Yield calculated from fraction R.4 F.3. ww. Yield calculated from fraction R.5 F.3. xx. Yield calculated from fraction 1. yy. Stromata obtained by centrifugation at 25,000 r.p.m. in the Sharples of aqueous suspensions of erythrocytes and retention of the precipitated material. Precipitate washed until essentially free of hemoglobin, centrifuging it at 5000 r.p.m. after each washing. zz. Yield calculated from lyophilized erythrocytes. aaa. Serological activity of a suspension prepared by dilution of an ethanol solution with physiological salt solution. bbb. The analytical data reported are the mean results of duplicate or triplicate analyses and are given together with the average deviation.

isolated by the less involved procedures, as indicated by the three criteria of activity discussed in the "Experimental" part above.

Kabat and co-workers have reported (21, 22) that the A-substances isolated from commercial hog gastric mucin by the 90% phenol extraction procedure of Morgan and King (2) and by subsequent purifications are about 60% pure on the basis of specifically precipitable hexosamine. Their report is based on the comparative results of a quantitative precipitin test (4) in which A-substance preparations derived only from serologically active hog gastric mucosae were found to participate to the extent of 100% of the hexosamine present. Our experiments indicate that obtaining substances more active than fractions 128 (alcohol fractionation out of water), R.2 F.2A (sodium sufate fractionation out of water), and 31 (phenol extraction with subsequent alcohol fractionation) is not unique to the use of hog gastric mucosae from "secretor" hogs. By the procedures outlined in Table I significant amounts of such more active materials can be obtained from Wilson hog gastric mucin also. We do not believe that our findings with respect to the possiblity of isolating highly active fractions from commercial hog mucin are in any way at variance with the findings of Kabat and co-workers (21, 22) that some hog gastric mucosae contain A-substance while others do not, and that in the latter mucosae there is material which is superficially similar to A-substance, but which has no significant serological activity. Thus, although the commercial hog gastric mucin with which we and others have worked may contain such material, the fact that no detailed information is available about the structure of either material leaves entirely open the question of what techniques may suffice to separate them.

In much of our preparative work we have used the technique of electrodialysis and have observed certain phenomena which we believe are significant. When an A-substance preparation is electrodialyzed under a potential gradient of ca. 18 volts/cm., a solid separates in the cell. This precipitation seems to be associated with an increase in the hydrogen ion activity of the cell and, ultimately, a pH of 4 or less may be attained. Accumulation of the precipitated solid begins near the cellophane membrane separating the central compartment from the anode compartment. The material which is precipitated can be dispersed or dissolved by the addition of alkali. In addition to the A-substance which is present in the precipitate there is a considerable amount dissolved in the clear or only slightly opalescent portion of the solution which overlays the turbid phase. Where comparison has been made between the serological activity of a precipitate obtained by electrodialysis and that of the material dissolved in its supernatant, it has been found that the more soluble fraction is 20-25% more potent in inhibiting hemolysis than the less soluble fraction, in the range where both activities are low (cf. fractions 111 and 112) and also where both are high (cf. fractions 66 and 68 and fractions 69 and 71). A-substance preparations which give rise in part to precipitated material when they are electrodialyzed cannot be regarded as homogeneous.

The so-called "neutral polysaccharide" (fraction 47), obtained by the procedure of Meyer, Smyth and Palmer (5), in which the original mucin is heated with 2% sodium carbonate for 15 minutes at 70°C. and from which an "acidic polysaccharide" is removed in acetic acid solution as a gelatin salt, has a very high potency in the inhibition of hemolysis test (see Table I), in accord with the findings of Landsteiner as reported by Meyer

et al. (5). Its activity in this test is greater than that of any other fraction obtained by us from hog gastric mucin with the single execption of R.7 F.5A which was obtained by heating another A-substance preparation in formamide solution at 150°C. (3). The equivalent N-acetylglucosamine content of fraction 47 is also higher than that of any other fraction except R.7 F.5A. We have found, however, that the potency of fraction 47 and the closely related fraction 48 in the inhibition of isoagglutination test is very low, and, thus, is in no way commensurate with the hemolysis inhibition activity. This indication of alteration in A-substance in the course of Meyer's procedure (5) has not previously been recognized, although the findings of Morgan and King (2) with respect to the lability of A-substance in alkaline solution strongly suggest that the initial alkaline treatment used by Meyer et al. might profoundly alter the serological properties of the product.

The relative serological activities of fractions R.6 F.5A and R.7 F.5A compared with those of the fractions from which they were derived (see Table I) confirm the findings of Landsteiner and Harte (3) and of Morgan and King (2) with respect to the effects of treating A-substance with papain-HCN or with formamide at 150°C. By both of these procedures the activity of the A-substance in inhibiting hemolysis is increased and in inhibiting isoagglutination is decreased.

We have found that the alcohol fractionation procedure of Landsteiner and Harte (3) with some modifications possesses advantages over other procedures for the preliminary concentration of A-substance from commercial (Wilson) hog gastric mucin for the purpose of further purification and investigation, conveniently providing an undegraded preparation which in

yield and potency is equivalent or superior to those obtained by other procedures. The desirable modifications in the Landsteiner and Harte procedure are: a, omission of the initial heating at 100°C.; and b, substitution of a thorough centrifugation in the open bowl of the Sharples of the aqueous suspension of mucin at a pH of 4.5. Centrifugation as indicated serves to remove about 15% of the weight of the mucin granules taken. The fraction which is removed at 40% (v/v) alcohol concentration is obtained in 35% yield and is 40-50% less active in inhibiting hemolysis than a centrifuged suspension of mucin. The most active fraction is obtained in 20% yield from mucin and has essentially twice the inhibition of hemolysis potency of crude mucin.

Summary

A study has been made of the relative merits of all previously reported procedures and of some new variants of these in isolating blood group A-specific substance from Wilson hog gastric mucin. In this study it has been possible, for the first time, to make direct comparisons of the serological activities of the fractions arising from all of these procedures. No fraction had more than 2-4 times the activity in inhibiting isoagglutination or 2-3 times the activity in inhibiting hemolysis of a centrifuged, undialyzed suspension of the original mucin. By rather complex procedures materials were obtained which had about 20% more activity in inhibiting hemolysis than those derived by any one of the hitherto described techniques.

Observations are reported on the isolation of A-substance from human erythrocytes and ovarian cyst fluids, and from hog pepsin.

References

1. Wiener, A.S., Blood Groups and Transfusion, 3rd ed., C. C Thomas, Springfield, Ill. 1946.

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

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

4. Kabat, E.A., and Bezer, A.E., J. Exptl. Med. 82 207 (1945).

5. Meyer, K., Smyth, E.M., and Palmer, J.W., J. Biol. Chem. 119 73 (1937).

6. Landsteiner, K., and Chase, M.W., J. Exptl. Med. 63 813 (1936).

7. Hallauer, C., Z. Immunitäts. 83 114 (1934).

8. Submitted for publication.

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

10. Kossjakow, P.N., Z. Immunitäts. 99 221 (1941).

11. Bray, H.G., Henry, H., and Stacey, M., Biochem. J. 40 124 (1946).

12. Calvin, M., Evans, R.S., Behrendt, V., and Calvin, G., Proc. Soc. Exptl. Biol. Med. 61 416 (1946).

13. Schiff, F., and Adelsberger, L., Z. Immunitäts. 40 335 (1924).

14. Landsteiner, K., and van der Scheer, J., J. Exptl. Med. 41 427 (1925).

15. Belkin, R.B., and Wiener, A.S., Proc. Soc. Exptl. Med. 56 214 (1944).

16. Yosida, K., Z. ges. exptl. Med. 63 331 (1928).

17. Morgan, W.T.J., and Van Heyningen, R., Brit. J. Exptl. Path. 25 5 (1944).

18. King, H.K., and Morgan, W.T.J., Biochem. J. (Proc.) 38 x (1944).

19. Morgan, W.T.J., and Waddell, M.B.R., Brit. J. Exptl. Path. 26 387 (1945).

20. Freudenberg, K., Westphal, O., Marriott, G., Groenewoud, P., and Molter, H., Sitzber. heidelberg. Akad. Wiss., Math.-naturw. Klasse (1938), Abhandl. 1.; Chem. Abstracts 35 3274 (1941).

21. Kabat, E.A., Bendich, A., and Bezer, A.E., J. Exptl. Med. 83 477 (1946).

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

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

III Spectrophotometric Study of Blood Group A Substance Isolated from Hog Stomach Sources

One of the necessary conditions for the proof of homogeneity of a high molecular weight substance is that its composition should not vary when successive fractionation procedures are applied. Of the methods available for following changes in composition on fractionation of A-substance, total nitrogen and N-acetyl glucosamine analyses, optical rotation, viscosity, and serological tests involving inhibition of isoagglutination of blood group A cells or hemolysis of sheep cells have been used (4, 19, 24). It is apparent that progress on the isolation of homogeneous preparations is achieved by applying many chemical, physical, and biological tests to the fractions obtained in the course of isolation. In this section the results of a study of the ultraviolet absorption spectra of fractions isolated from mucin, pepsin, and individual hog stomach linings by a variety of procedures are reported. The spectra provide evidence for the existence and nature of possible contaminants as well as qualitative information concerning the importance of aromatic amino acids in A-substance.

The ultraviolet absorption spectra of preparations of blood groups O, A, and B substances from human urine and A-substance from hog stomachs isolated by Freudenberg (8) were first studied by Gróh, Szélyes, and Weltner (9) and in greater detail by Weltner (30). In neutral aqueous solution all the blood group substances showed weak selective absorption in the region 270-290 mu. It would appear that purification of A-substance from hog stomachs caused the extinction to increase throughout the ultraviolet region since a "purified" preparation and a "highly purified" preparation showed extinction

coefficients, $E^{1\%}_{1cm}$, at 280 mu of 1.8 and 2.8 respectively. These results are at variance with those we have obtained from A-substance from various hog stomach sources; preparations with greatly enhanced potencies show little absorption in the ultraviolet.

Experimental

Apparatus. The Beckman Model DU spectrophotometer with quartz cells was used (5). Optical densities for 1 cm layers of solution at about 25°C were usually measured at every 2 mu from 220 to 270 mu, every 5 mu from 270 to 300 mu and every 10 mu from 300 to 350 mu. Additional points were taken when spectra showed maxima shifted from the usual 260 mu maximum. The spectra for solutions were corrected for the absorption due to the solvent (citrate-phosphate buffer) which was important below 240 mu. Extinction coefficients, $E^{1\%}_{1cm}$, were calculated from the usual relation, $E^{1\%}_{1cm} = (\log_{10} I_o/I)/cd$, where d is the cell width (1 cm) and c is the concentration of solute in grams per 100 ml of solution.

A-substance solutions. Most of the A-substance preparations have been described previously (4, 15). Notes to Table II describe briefly the method of preparation.

The solutions were prepared by dissolving preparations in distilled water, taking 10 ml aliquots and adding 1 ml of McIlvaine's citrate-phosphate buffer pH 7; the pH of the resulting mixture was 7.20 ± 0.05 and the A-substance concentration varied from 300 to 380 micrograms/ml. In a few instances, where indicated, solutions were prepared similarly at pH 6.30 ± 0.05 and pH 8.05 ± 0.05 by

means of McIlvaine buffers. The final citric acid and Na_2HPO_4 concentrations of the solution at pH 6.3, 7.2, and 8.0 were respectively 0.0034 M $H_3C_6H_5O_7$ - 0.0115 M Na_2HPO_4, 0.0016 M $H_3C_6H_5O_7$ - 0.015 M Na_2HPO_4, and 0.0003 M $H_3C_6H_5O_7$ - 0.0176 M Na_2HPO_4.

Serological tests. The inhibition of isoagglutination test (15) and the inhibition of hemolysis test (4) have been described previously; the activities of many of the preparations were reported in the above papers.

Results and Discussion

A-substance preparations dissolved in distilled water generally give clear or slightly opalescent solutions. The solutions are colorless for undegraded preparations; degraded products may give slightly yellowish solutions. The solutions depart considerably from ideality as may be judged from the failure of Beer's law shown in the data in Table I. The extinction coefficients at a given wavelength and pH increase on dilution. Futhermore the extinction values show a strong dependence on pH; the extinction values generally increase with decreasing pH in the region 220 to 350 mu.[1] The extinction curves in the range pH 6 to 8 are not altered on standing. The

---

[1] The change in extinction values with pH may have a contributing factor due to the difference in salt concentration in the various buffer solutions. Results (Part V) indicate that ionic strength also influences spectra.

Table I

Effect of pH and A-substance Concentration on

Extinction Values at Various Wavelengths

Fraction	A-substance concentration micrograms/ml	pH	Extinction Coefficients $E^{1\%}_{1cm}$ 220 mu	240 mu	260 mu	300 mu	350 mu
R1-F2A	89.1	6.3	27.5	3.1	2.2	1.0	0.6
	89.1	7.2	27.0	2.8	1.9	.8	.4
	89.1	8.0	26.6	2.3	1.4	.4	.2
	445	7.2	26.5	2.2	1.5	.7	.3
R2-F2A	53.8	6.3	25.3	2.8	2.0	.4	
	53.8	7.2	24.4	2.3	1.3	.6	
	53.8	8.0	23.7	2.3	1.4	.4	
	269	7.2	23.9	1.9	1.3	.4	.1
R4-F3	69.5	6.3	37.6	13.3	13.5	3.9	2.4
	69.5	7.2	34.5	9.0	10.8	1.2	.7
	69.5	8.0	32.2	8.0	9.7	.8	.4
	347	7.3	29.7	6.1	6.0	1.6	1.0
R5-F3	86.5	6.3	35.6	11.2	11.2	3.6	2.1
	86.5	7.2	32.3	7.3	8.7	1.6	.8
	86.5	8.0	31.9	7.3	8.1	.9	.6
	433	7.3	31.3	7.2	8.0	1.4	.8
R6-F5A	64.4	6.3	22.0	3.1	2.4	.8	.5
	64.4	7.2	21.5	2.0	1.3	.5	.4
	64.4	8.0	20.8	1.8	.7		
	322	7.2	19.5	1.3	.6	.2	
R7-F5A	37.8	6.3	19.3				
	37.8	8.0	25.0	3.8			
	94.5	6.3	21.8	1.5	1.0	.4	.2
	94.5	8.0	22.0	2.4	1.5	.5	.3
	189	6.3	22.8	2.8	1.9	.8	.4
	189	7.2	22.9	2.4	1.7	.7	.4
	189	8.1	21.7	1.9	1.3	.3	.1

stability of the solutions at these pH is in agreement with other observations by Morgan and King (24) and Kabat, Bendich and Bezer (18). Weltner (30) observed that extinction curves in 0.1 N NaOH showed a continual increase with time, a fact which may now be interpreted as due to the recognized lability of A-substance in alkaline solution (24). Due to the dependence of extinction values on pH and A-substance concentration the spectra of preparations which are discussed below were measured in solutions at pH 7.2 and at similar concentrations.

1. Extinction Curves of Preparations from Hog Gastric Mucin

Commercial hog gastric mucin[1], centrifuged free of inactive water-insoluble material shows strong selective absorption in the ultraviolet at 260 mu (C-135, Fig. 1); appreciable apparent absorption occurs even at 350 mu. The latter absorption is due to light scattering since mucin is visibly turbid and the apparent absorption extends far into the visible where specific absorption is unlikely. The absorption above 330 mu possesses a fairly constant Rayleigh coefficient of 2.5 - 3.5 over a considerable wavelength range which presumably is typical for non-specific absorption (16, 17). The material absorbing at 260 mu in mucin ("260 mu component") is non-dialyzable as may be seen from C-125 which represents a dialyzed suspension.

---

[1] Wilson Laboratory hog gastric mucin granules, Item No. 443.

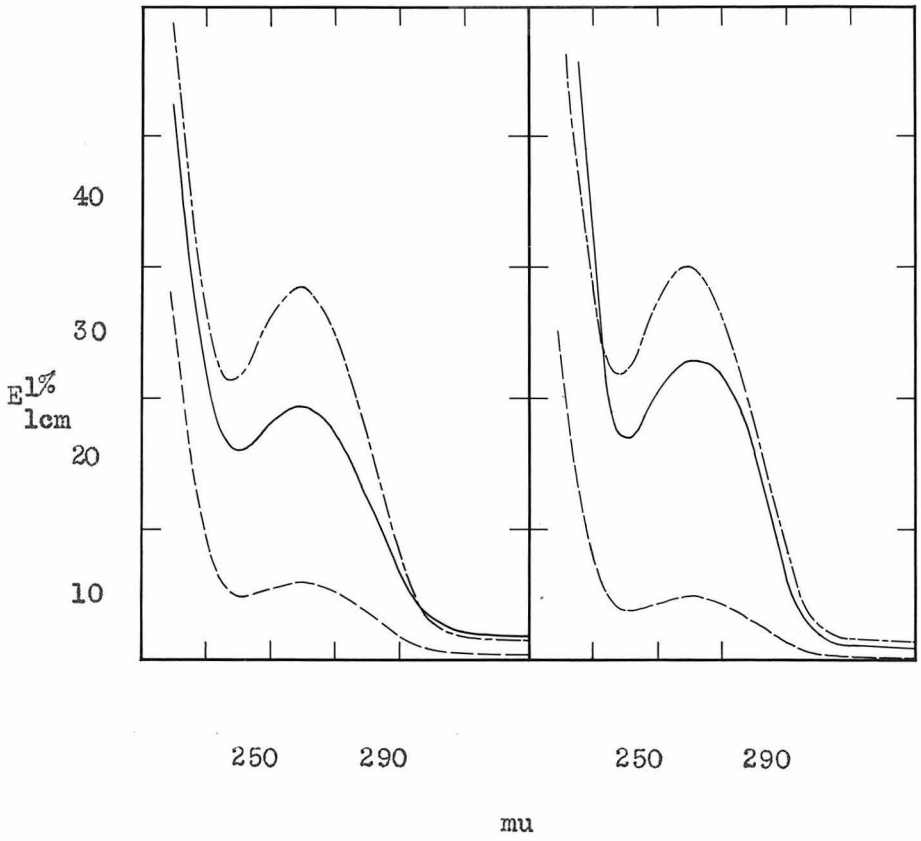


Fig. 1 . Extinction curves of fractions isolated from hog gastric mucin by alcohol fractionation.
Left: - ——— - , C-123; ——— , C-125; —— ——, C-128.
Right: - ——— - , C-142; ——— , C-135; —— ——, C-143.

The 260 mu component has not been well characterized so far since it appears to be associated with inactive or less active fractions isolated from mucin. The presence of nucleic acid is suggested by the position of the peak (10, 21); Bray, Henry and Stacey (2) have recently reported that mucin may contain nucleic acid of mixed ribose and desoxy ribose type. However preparations (0.04% solutions) showing the 260 mu component do not give the Dische cysteine test (7) for desoxy pentose. Weakly positive orcinol tests for pentose (22) are given but these results may not be significant since hexoses, which are amply present in mucin, also are known to give weak orcinol tests (26). The 260 mu component can not be centrifuged out by high speed centrifugation since after centrifuging at 18,000 RPM for thirty minutes (40,000 x G) the specific absorption remains; a slight decrease in extinction occurs throughout the ultraviolet due to the separation of a small amount of suspended material and the resulting clarification of the solution (Fig. 2)[1]. Preliminary experiments indicate that the 260 mu component can be readily modified by heating with acid, alkali, or by the action of oxidizing agents such as hypoiodite.

In the course of evaluating the usefulness of spectrophotometric analyses in detecting contaminants and following changes in composition on fractionation, the extinction curves for many active

---

[1] R18-F2 and R18-F12 are fractions obtained by alcohol fractionation and are discussed in Part IV.

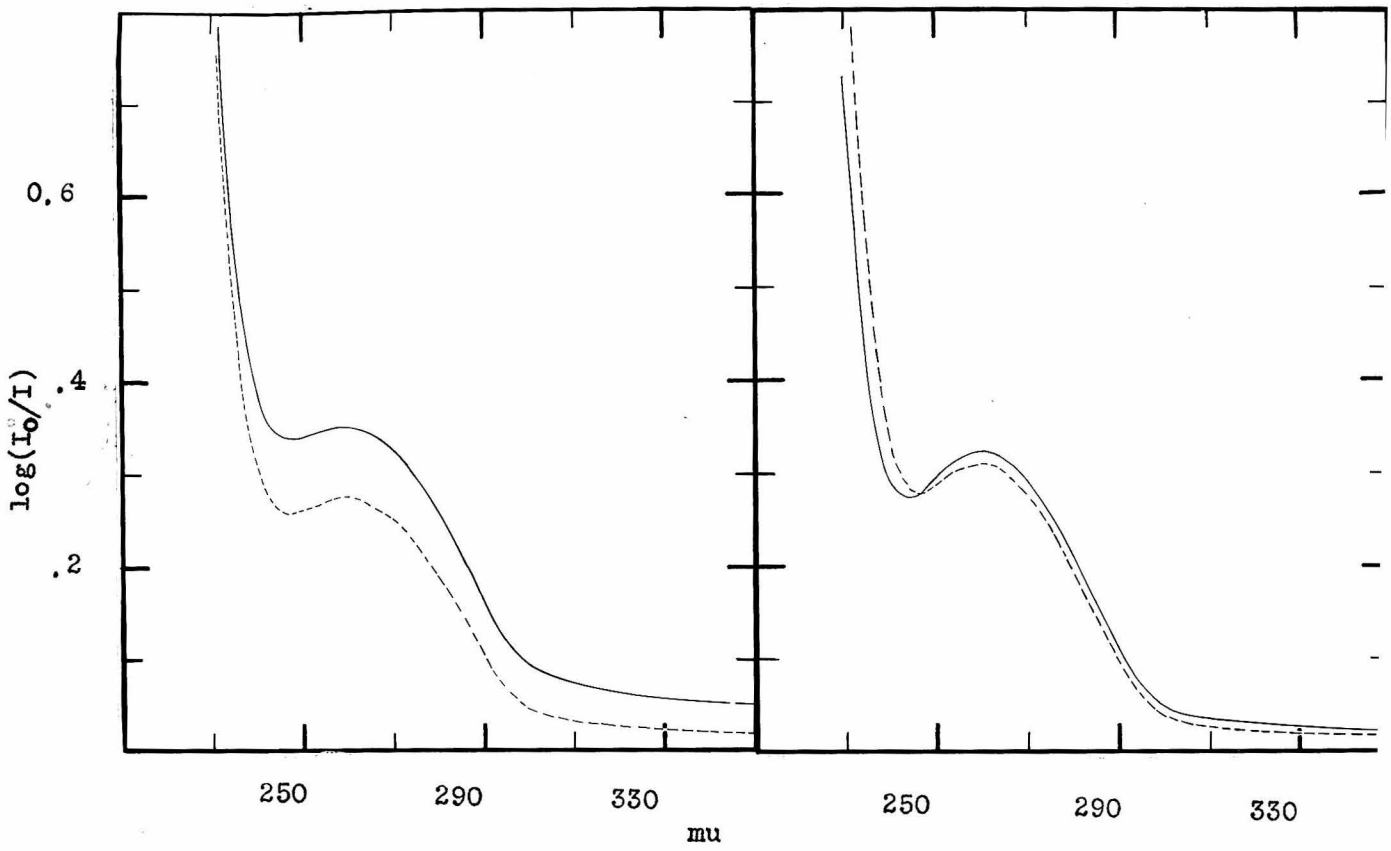


Fig. 2 . Absorption spectra of uncentrifuged fractions (solid curves) and centrifuged fractions (dotted curves) obtained by alcohol fractionation from hog gastric mucin. Left: R18-F12, 927 γ/ml. Right: R18-F2, 915 γ/ml.

and inactive fractions from various procedures were measured. Typical extinction curves are shown in Figs. 3 - 8 and in Table II the serological properties and the extinction values at 260 mu and 350 mu are summarized.

On fractionation of an aqueous centrifuged mucin suspension with alcohol (19) the more active fractions which are soluble in 40% alcohol and insoluble in 60 - 65% alcohol always show considerably less absorption throughout the ultraviolet region than the original mucin suspension (C-128 and C-143, Fig. 1; R4-F3 and R5-F3, Fig. 3). The 260 mu component still occurs in these fractions since $E^{1\%}_{1cm}$ varies from 5 to 8. The sensitivity of the spectrophotometric method may be appreciated since the four fractions prepared by the same method have essentially the same serological activity but differ significantly in their extinction curves. On the other hand the 260 mu component is concentrated in the fraction insoluble in 40% alcohol (C-123 and C-142, Fig. 1; R4-F2 and R5-F2, Fig. 3). A new component is detectable in the fraction soluble in 60% alcohol since absorption occurs maximally in this case at about 270 mu (R4-F4, Fig. 3). This fraction which possesses essentially no serological activity contains considerable free amino acids; in one instance about 2% amino acid nitrogen was detectable by the Van Slyke manometric method.

The products isolated from hog gastric mucin by Morgan and King (24) by 90% phenol extraction followed by precipitation with alcohol or by aqueous extraction followed by precipitation with sodium sulfate were not distinguishable by a number of different tests including serological activity, elementary analysis, optical rotation

Table II

Spectral and Serological Properties of Fractions Isolated from Hog Gastric Mucin, Pepsin, and Individual Hog Stomach Linings

Procedure	Fraction	$E^{1\%}_{1cm}$ 260 mu	$E^{1\%}_{1cm}$ 350 mu	Inhibition of isoagglutination titer ul B serum neutralized per γ	Inhibition of hemolysis titer γ inhibiting 50% hemolysis
Aqueous suspension of mucin fractionated with ethanol after centrifuging twice at pH 4.4 in Sharples.	C-125	19.4	1.7	45 ± 15	0.18 ± .01
	C-123	28.5	1.5	40 ± 15	.24 ± .01
	C-128	5.9	.5	75 ± 20	.11 ± .00
	C-135	22.8	.9	45 ± 15	.18 ± .02
	C-142	30.1	1.3	45 ± 15	.24 ± .00
	C-143	4.9	.3	85 ± 20	.11 ± .01
Alcohol fractionation of mucin after procedure of Landsteiner and Harte (19).	R4-F2	30.4	2.2	30 ± 10	
	R4-F3	6.0	1.0	80 ± 20	.12 ± .01
	R4-F4	31.2	1.3	.1 ± .05	
	R5-F2	41.9	3.2	20 ± 10	
	R5-F3	8.0	.8	85 ± 20	.11 ± .01
Alcohol fractionation of a centrifuged 90% phenol solution of mucin (24) followed by electrodialysis and alcohol fractionation.	C-52	11.6	1.0	50 ± 15	.13 ± .01
	C-59	1.7	.5	90 ± 30	
	C-61	2.2	.6	50 ± 15	
Sodium sulfate fractionation of aqueous centrifuged suspension of mucin (24) followed by electrodialysis.	R2-F2A	1.3	.1	45 ± 15	.11 ± .01
	R2-F2B	2.1	.3	50 ± 15	
	R2-F2C	3.8	.9	50 ± 15	
Electrodialysis of product of alcohol fractionation of mucin, R5-F3.	R8-F1	1.4	.2	60 ± 20	
	R8-F2B	7.6	2.1	50 ± 15	

Table II.-(concluded)

Procedure	Fraction	$E^{1\%}_{1cm}$ 260 mu	$E^{1\%}_{1cm}$ 350 mu	Inhibition of isoagglutination titer ul B serum neutralized per γ	Inhibition of hemolysis titer γ inhibiting 50% hemolysis
Treatment of products of alcohol fractionation of mucin with ion-exchange resins.	C-110	13.2	0.5		0.20 ± .02
	C-165	11.8	.6		.20 ± .02
	C-167	3.6	.5		.21 ± .02
	C-168	3.4	.5		.07 ± .005
Treatment of product of alcohol fractionation of mucin with papain-HCN (19).	R6-F5A	.6	.1	25 ± 10	.10 ± .01
Treatment of product of alcohol fractionation of mucin with formamide at 150°C (19).	R7-F5A	1.7	.4	10 ± 5	.05 ± .005
Alkaline digestion of mucin followed by selective adsorption and precipitation (25).	C-47	.8	.2	30 ± 10	.065 ± .01
Alcohol fractionation of Wilson pepsin (20).	C-22	3.7	.4	70 ± 15	.05 ± .005
Autolysis of Wilson pepsin followed by alcohol fractionation (19).	C-26	5.0	.3	35 ± 10	.04 ± .005
Peptic digestion of individual hog stomach linings followed by two alcohol precipitations (3)[1].	H1-F3	3.6	.3	< .06	> 175
	H2-F1	4.7	.3	90 ± 30	.08 ± .005
	H2-F3	2.9	.2	.5 ± .2	.60 ± .05
	H3-F1	7.3	.3	75 ± 20	.13 ± .01
	H3-F2	4.4	.2	2 ± 1	.70 ± .05
	H4-F1	9.6	.3	< .06	> 175

1 Extinction curves were measured on solutions containing about 900 micrograms of compound per ml rather than the usual 300-350.

Notes to Table II.- C-125: Material from dialyzed centrifugate. C-123: Material insoluble in 40% (v/v) ethanol. Dialyzed. C-128: Material soluble in 40% (v/v) ethanol but insoluble in 65% (v/v) ethanol. C-135: Material from undialyzed centrifugate. C-142: Material insoluble in 40% (v/v) ethanol. Not dialyzed. C-143: Material soluble in 40% (v/v) ethanol, but insoluble in 65% (v/v) ethanol. Not dialyzed. R4-F2: Insoluble on addition of 3/4 vol. ethanol to filtrate of aqueous suspension of mucin. R4-F3: Precipitated between 3/4 and 2 vol. ethanol from filtrate of aqueous suspension of mucin. R4-F4: Soluble material after addition of 2 vol. ethanol to filtrate of aqueous suspension of mucin. R5-F2: Same as R4-F2, duplicate experiment. R5-F3: Same as R4-F3, duplicate experiment. C-52: Material insoluble in 10% (v/v) ethanol. C-59: soluble in 47% (v/v) ethanol and insoluble in 62% (v/v) ethanol on fractionation of clear supernatant obtained on electrodialysis of C-52. Dialyzed. C-61: Insoluble in 47% (v/v) ethanol on fractionation of clear supernatant obtained on electrodialysis of C-52. R2-F2A: Insoluble in 30% sodium sulfate. Precipitate electrodialyzed. Material from supernatant. R2-F2B: Aqueous washings of R2-F2C. R2-F2C: Precipitate obtained on electrodialysis of 30% sodium sulfate precipitate. R8-F1: Clear supernatant on electrodialysis of R5-F3 for five days. R8-F2B: Residue on electrodialysis of R5-F3 for five days. C-110: Material soluble in 30% (v/v) ethanol but insoluble in 65% (v/v) ethanol from centrifuged mucin suspension. Upon reprecipitation insoluble in 45% (v/v) ethanol. Electrodialyzed. C-165: C-110 treated twice with alkali-washed IR-4 resin. C-167: C-110 treated twice with alkali-washed Deacidite resin. C-168: Material soluble in 45% (v/v) ethanol and insoluble in 65% (v/v) ethanol from centrifuged suspension, dialyzed, and then treated twice with alkali-washed Deacidite resin. R6-F5A: Corresponds to preparation III of Landsteiner and Harte (19). R7-F5A: Corresponds to preparation II of Landsteiner and Harte (19). C-47: Material not precipitated by $Zn(OH)_2$ at pH 7.1 but precipitated by acetone-acetic acid. Material from supernatant after electrodialysis of final precipitate. C-22: Autolyzed pepsin, precipitated by 62% (v/v) ethanol and then by 9 vol. glacial acetic acid and 5 vol. acetone; dialyzed and precipitated by 4 vol. ethanol plus sodium acetate. C-26: Obtained by two 65% (v/v) ethanol precipitations at pH 8.5, dialyzed. H1-F3: Soluble in water and insoluble in 71% (v/v) ethanol. H2-F1: Soluble in water and insoluble in 63% (v/v) ethanol. H2-F3: Soluble in 63% (v/v) ethanol and insoluble in 74% (v/v) ethanol. H3-F1: Soluble in water and insoluble in 63% (v/v) ethanol. H3-F2: Soluble in 63% (v/v) ethanol and insoluble in 71% (v/v) ethanol. H4-F1: Soluble in water and insoluble in 71% (v/v) ethanol.

or viscosity. However, we have found that extinction curves of A-substance fractions prepared according to the two procedures are readily distinguished. The product from the phenol-alcohol procedure contains greater amounts of the 260 mu component (C-52, Fig. 4) than the A-substance isolated by alcohol fractionation. The product isolated by the sodium sulfate procedure would appear to have less of the 260 mu component (Fig. 4); the fraction corresponding to the sodium sulfate product of Morgan and King would be represented by an extinction curve roughly intermediate between R2-F2A and R2-F2C since the Morgan and King product was fractionated further as discussed below.

It was reported earlier (4) that A-substance fractions obtained by alcohol, sodium sulfate, or phenol-alcohol fractionation could be shown to be heterogeneous by electrodialysis. Solid material separated in the cell during electrodialysis leaving a fairly clear supernatant. The residue and the material in the supernatant possessed approximately the same equivalent N-acetyl glucosamine content and the same inhibition of isoagglutination activity. In the more precise inhibition of hemolysis test the material in the supernatant appeared to be 20-25% more active than the residue. While these two fractions are quite similar by these criteria they differ substantially in their spectral properties. The solid disperses in water to give quite turbid solutions while the material in the supernatant yields only slightly turbid solutions. The latter solution, of course, scatters considerably less light in the ultraviolet as may be seen from the extinction in the region 300-

350 mu. In Fig. 4, R2-F2A, R2-F2B, and R2-F2C represent respectively the material in the supernatant, a washing of the residue, and the residue. R8-F1 and R8-F2B (Fig. 5) are respectively the supernatant material and the residue on electrodialysis of the main product, R5-F3, obtained by alcohol fractionation of mucin. C-59 and C-61 (Fig. 4) are the soluble materials obtained on electrodialysis of the product of phenol-alcohol fractionation, C-52. C-59 and C-61 differ in that the former is soluble in 47% alcohol and insoluble in 60% alcohol while the latter is insoluble in 47% alcohol. In every case the soluble materials, which are more active serologically than the residue or the parent compound, show decreased absorption in the ultraviolet. This would appear to be due in part to the fact that light-scattering components are concentrated in the residue but also because the 260 mu component occurs to a greater extent in the residue.

A study has been made in this Laboratory[1] of the use of ion-exchange resins in concentrating A-substance from hog gastric mucin. Preparations comparable in activity to those obtained by other procedures are obtained in this way. C-168 (Fig. 5) which exhibits weak absorption in the ultraviolet was obtained by treating a 60% alcohol precipitate from mucin twice with base-treated Deacidite. The 40% alcohol precipitate which contains considerable quantities of the 260 mu component (C-110) can be changed to a fraction showing weak 260 mu absorption by treatment with base-treated

---

[1] Unpublished work of E. L. Bennett.

Deacidite (C-167). Treatment of C-110 with base-treated IR-4 resin, a resin of similar type, is ineffective however in removing the 260 mu component (C-165).

A-substance is known to degrade under a variety of conditions, e.g., by heating with formamide at 150°C (19, 24), by digestion with papain-HCN (19), and by treatment with alkali (18, 24). Degradation is usually observed by a decrease in the potency in the isoagglutination test while no change or an increase in potency in the hemolysis test is observed. It was of interest to determine the extent to which the spectral properties were altered by degradation. Typical curves are shown in Fig. 6. All the degraded preparations show very little absorption in the ultraviolet and as a consequence of the clarity of the aqueous solutions low extinction values are observed in the 300-350 mu range. R6-F5A was obtained from R4-F3 by papain-HCN digestion (19), R7-F5A by heating R5-F3 with formamide at 150°C (19), and C-47 by preliminary digestion with 2% sodium carbonate at 70°C followed by adsorption and precipitation according to the procedure of Meyer, Smyth, and Palmer (25).

2. Extinction Curves of Preparations from Hog Pepsin

In Fig. 7 are shown extinction curves for fractions isolated by the procedure of Landsteiner and Chase (20), C-22, and by the procedure of Landsteiner and Harte (19), C-26, from Wilson pepsin. Unlike the active preparations from hog gastric mucin specific

absorption is shown in the region 270 mu possibly due to contamination from the considerable quantities of protein present in the starting material. These fractions are among the most active in the hemolysis test that have been isolated so far from hog stomach sources. Evidence has been presented previously (4) which suggests that these preparations may in fact be partially degraded. The extinction curves would indicate that despite their high potency they contain contaminants or artifacts produced in the course of isolation which absorb at 270 mu. Purified preparations from hog gastric mucin show considerably less absorption in the ultraviolet.

3. Extinction Curves of Preparations from Individual Hog Stomach Linings

In 1945 Witebsky (29) reported that only 40% of hogs have the specific blood group A factor in their stomach linings. In was later reported by Bendich, Kabat, and Bezer (3) that hog stomach linings may be differentiated into those possessing the A-factor and those having a similar but inactive mucoid. The latter material was shown by Aminoff, Morgan, and Watkins (1) to possess properties of blood group "O" substance. Hog gastric mucin, being a pool of stomachs of many hogs was shown to contain both A and O substances. It was, of course, of interest to compare the spectral properties of the A-substance derived from individual hog stomach linings with those isolated from hog mucin. Accordingly partially purified fractions were isolated from individual hog stomach linings by peptic digestion followed by alcohol fractionation. The extinction curves for these

preparations are shown in Fig. 8 and their serological properties in Table II. Hogs 1 and 4 gave inactive products, presumably O-substance, while hogs 2 and 3 possessed A-specific material. The activities of the more potent preparations were about the same order of magnitude as those obtained from mucin and pepsin. However all the preparations showed considerably more specific absorption in the region 260-270 mu than preparations from hog gastric mucin. The spectra of materials not possessing A activity, H1-F3 and H4-F1, resemble those possessing A activity, H2-F1 and H3-F1; the similarity parallels the observations of Bendich, Kabat and Bezer (3) as to the similar viscosity and elementary analyses of active and inactive materials. Attention may be also called to the similar extinction curves found by Weltner (30) for blood group O, A, and B substances from human urine. H2-F3 and H3-F2 represent fractions not previously observed in hog mucin fractionation; these fractions are soluble in 60% alcohol and insoluble at about 72% alcohol and have little activity compared to the fractions insoluble at 60% alcohol, H2-F1 and H3-F1.

4. Spectrophotometric Estimation of the Maximum Aromatic Amino Acid Content of A-substance Preparations

Spectrophotometric procedures for determining tyrosine and tryptophan in proteins have been reported by Holiday (13, 14) and recently by Goodwin and Morton (11). The results of these procedures generally agree with those obtained by other methods if precautions in interpretation are appreciated (23) and if suitable corrections

for non-specific absorption are made. The methods depend on the fact that of all common natural amino acids only tyrosine, tryptophan and phenylalanine show absorption in the region 260-300 mu. Since phenylalanine shows considerably less absorption than either tyrosine or tryptophan in this region it is usually neglected when analyzing for the other amino acids.

At pH 7.2 in phosphate-citrate buffer it was found that tryptophan has a maximum at 280 mu, $E^{1\%}_{1cm} = 278$, and a minimum at 244 mu, $E^{1\%}_{1cm} = 83$; tyrosine has a maximum at 274 mu, $E^{1\%}_{1cm} = 78$, and a minimum at 245 mu, $E^{1\%}_{1cm} = 11$; phenylalanine shows weak absorption at 255 mu, $E^{1\%}_{1cm} < 5$; and histidine shows specific absorption only below 240 mu. A-substance is known to contain N-acetyl glucosamine and galactose (20, 25) and recently fucose and mannose have been detected (2). These sugars would be expected to show ultraviolet absorption only below about 230 mu (12, 21). If the extinction of A-substance at 280 mu after correction is ascribed solely to amino acids then it is possible to estimate the maximum content of tryptophan or tyrosine in these preparations. Since the preparations absorb weakly in the ultraviolet and because tyrosine and tryptophan possess similar absorption spectra under the test conditions, it is difficult to determine tyrosine and tryptophan simultaneously as has been done previously (11, 13). The procedure of measuring absorption in alkaline solution which allows considerable differentiation in the tyrosine and tryptophan spectra is inapplicable due to the known lability of A-substance.

The equivalent tyrosine or tryptophan content of a number of

preparations have been calculated and are summarized in Table III. The method adopted here has been to calculate the tyrosine or tryptophan content by ascribing the net absorption at 280 mu entirely to either tyrosine[1] or tryptophan. Maximum values for these amino acids then are obtained. The extinction coefficient at 280 mu observed for the preparation is corrected for non-specific absorption in the ultraviolet by extrapolating from the extinction curve above 320 mu, a region in which even tyrosine and tryptophan are transparent. This correction is necessary and has been applied previously (11, 13) to protein analyses and agrees within experimental error with the correction which is found by determining the Rayleigh coefficient for the non-specific absorption and using the coefficient for extrapolation. If the correction for non-specific absorption is applied throughout the ultraviolet region then the more potent preparations which show low plateaus in the region 260-280 mu possess extinction curves which have small maxima between 270-280 mu.

While the preparations shown in Table III are probably not pure it seems certain that tyrosine and tryptophan if present in A-substance are among the least abundant amino acids. The maximum tyrosine is between .3-1% and the maximum tryptophan between 0.1-.3% for purified preparations from hog mucin; these estimates would of course be smaller if both of the amino acids were present. Less pure preparations from mucin and preparations from pepsin and hog linings

---

[1] $E^{1\%}_{1cm}$ for tyrosine at 280 mu is 69.

Table III

Calculated Maximum Tyrosine and Tryptophan Content

of A-substance Preparations

Fraction	$E^{1\%}_{1cm}$ * 280 mu	Correction for non-specific absorption 280 mu	$E^{1\%}_{1cm}$ corrected	Maximum percent tyrosine	Maximum percent tryptophan
R2-F2A	1.21	0.35	0.86	1.2	0.3
C-59	1.57	.85	.72	1.0	.25
R8-F1	1.30	.50	.80	1.1	.3
C-168	2.74	.92	1.82	2.6	.65
R7-F5A	1.24	.68	.56	.8	.2
C-47	.57	.30	.27	.4	.1
R6-F5A	.50	.20	.30	.4	.1
C-22	3.45	.70	2.75	4.0	.95
C-26	2.99	.66	2.33	3.4	.8

* Solutions in citrate-phosphate buffer, pH 7.2.

have larger apparent contents of tyrosine and tryptophan. Since molecular size studies (20, 29) indicate molecular weights of the order of 250,000 or larger the maximum tyrosine and tryptophan content represents respectively about 4-14 and 1-4 molecules per molecule of A-substance. The spectra of the A-substance preparations resemble proteins like gelatin (28) which also exhibits little absorption in the ultraviolet and possesses little tyrosine or tryptophan.

The results above are in agreement with qualitative tests for aromatic amino acids in A-substance which are generally weak or negative (19). The only quantitative results so far are those of Brand and Saidel (6) on a preparation of A-substance from hog gastric mucin by the phenol prodedure in which it is reported that 0.3% tyrosine and 0.2% tryptophan are present.

Summary

The results which have been presented indicate that spectrophotometric analysis is useful for following changes in composition on fractionation of A-substance from hog stomach sources as well as comparing preparations isolated by different methods and from different sources. Of particular advantage is that the method is fairly precise, requires small samples, and the analyses can be carried out rapidly and conveniently. The results on hog gastric mucin indicate that A-substance has little specific absorption in the ultraviolet; extraneous components account for most if not all the absorption. Purification has always led to decreases in absorption in the ultraviolet. A small amount of non-specific absorption is present in undegraded preparations due presumably to light scattering by the slightly opalescent solutions; degraded preparations show considerably less opalescence.

The maximum tyrosine and tryptophan content of preparations can be estimated by spectrophotometric analysis. The analyses indicate that the amino acids are minor constituents in the preparations from hog gastric mucin. Preparations from pepsin and individual hog stomach linings would appear to contain significantly more tyrosine and / or tryptophan, but the possibility of contaminants in these preparations is not excluded.

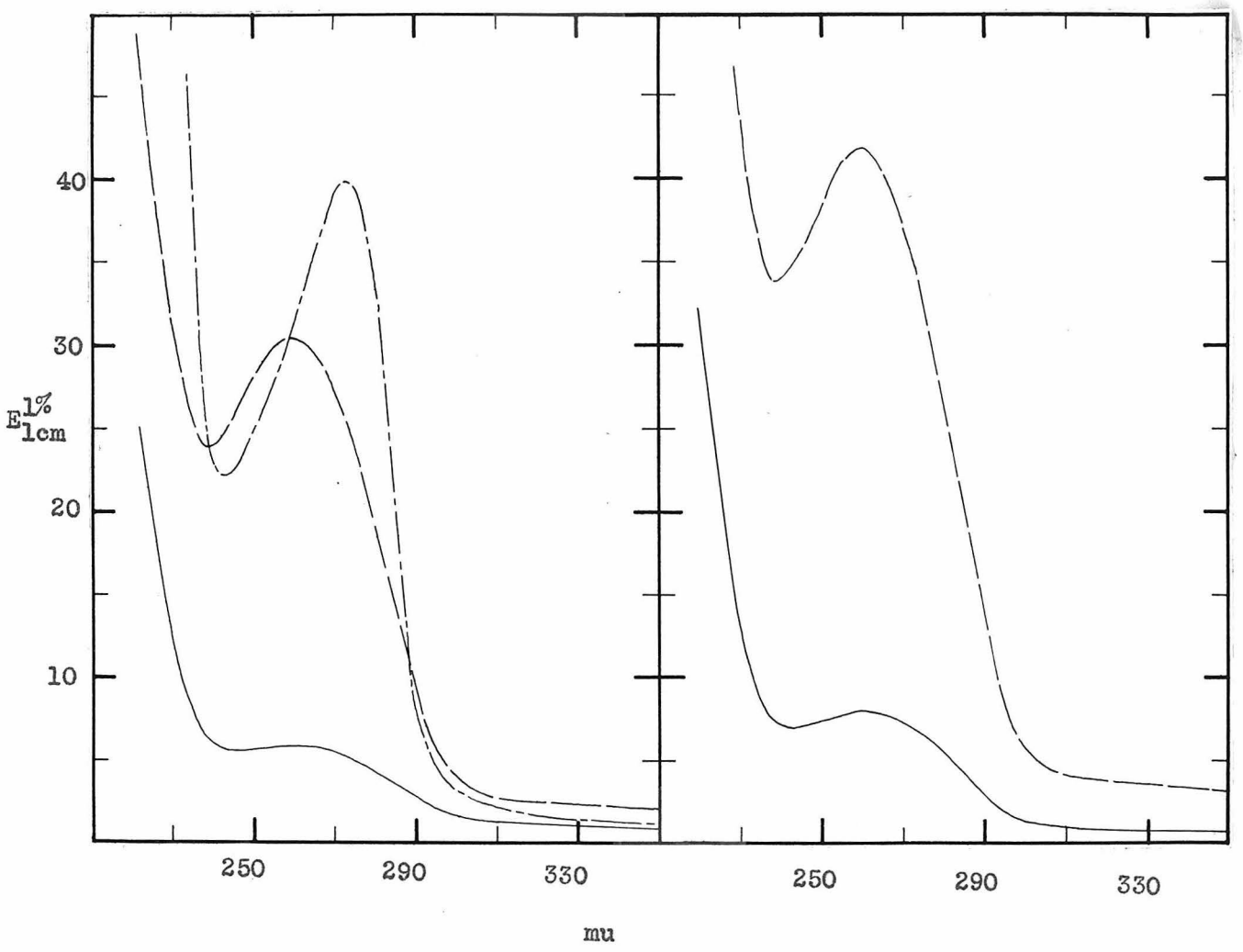


Fig. 3 . Extinction curves of fractions isolated from hog gastric mucin by alcohol fractionation. Left:- - - -, R4-F4; — — —, R4-F2;————, R4-F3. Right:— — —, R5-F2;————, R5-F3.

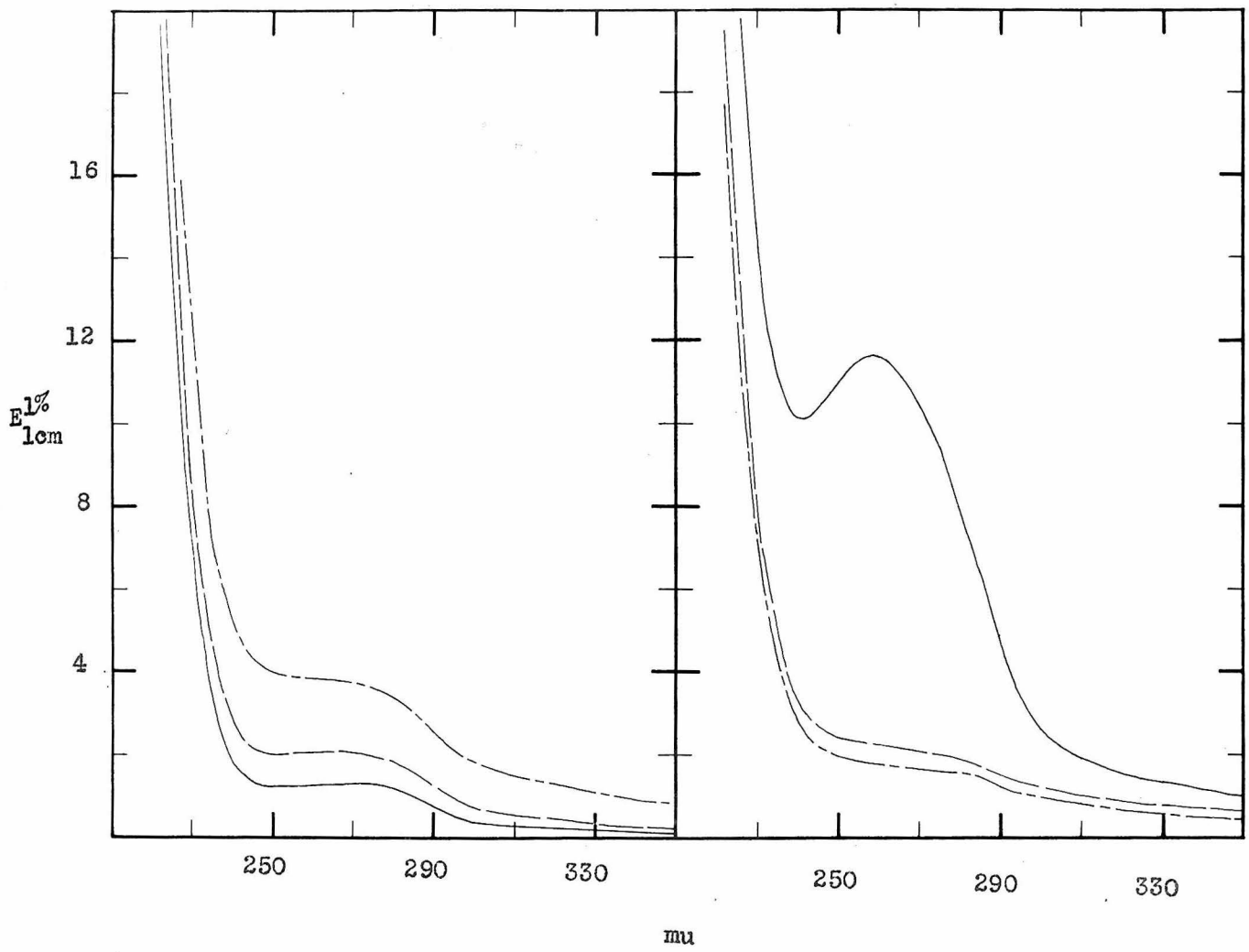


Fig. 4 . Extinction curves of fractions isolated from hog gastric mucin by sodium sulfate fractionation followed by electrodialysis (left) and by phenol-alcohol fractionation followed by electrodialysis and alcohol fractionation (right).
Left:– – – –, R2-F2C; — — —, R2-F2B; ————, R2-F2A.
Right: ————, C-52; — — —, C-61;– – – –, C-59.

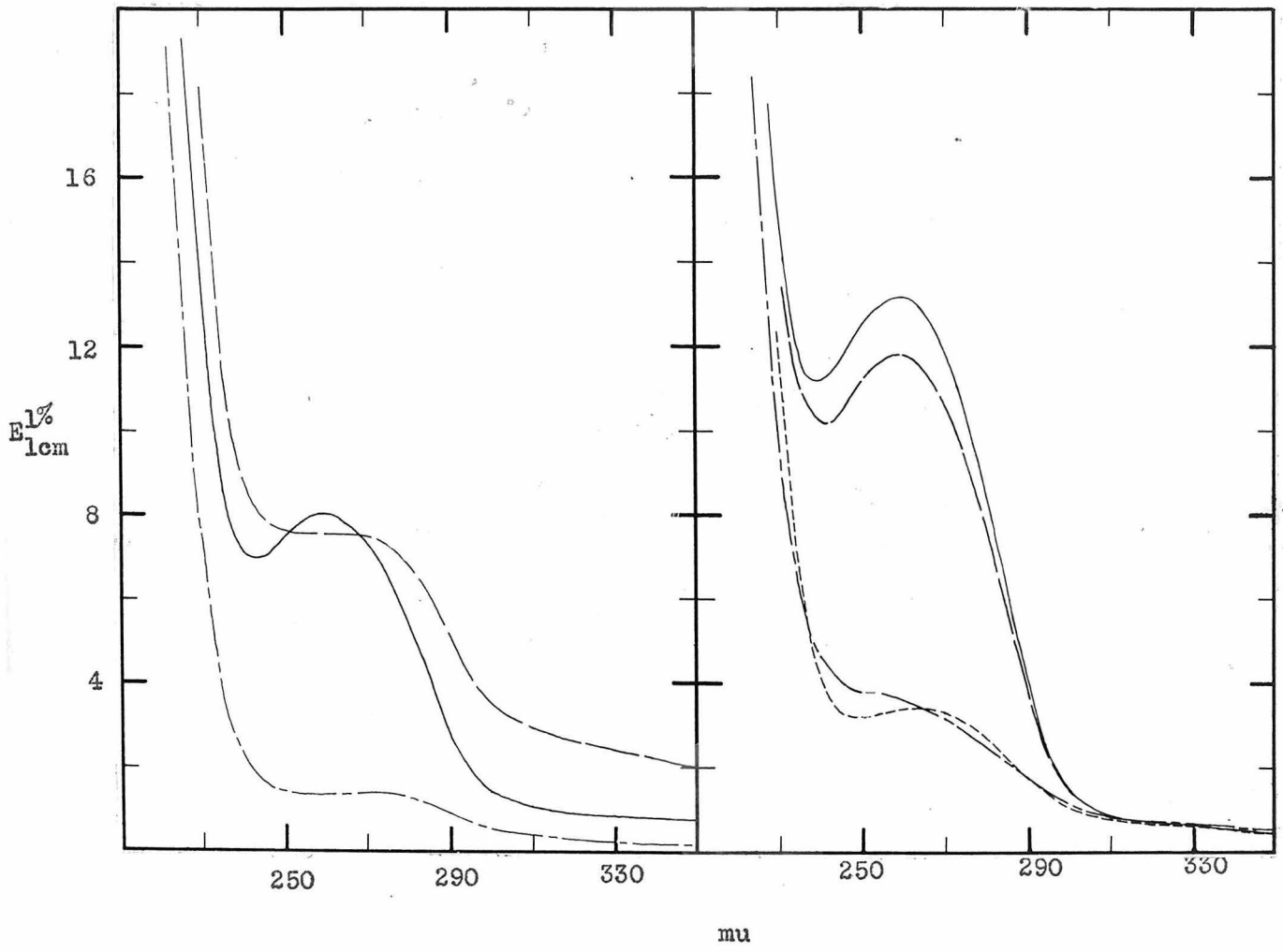


Fig. 5 . Extinction curves of fractions isolated from hog gastric mucin by alcohol fractionation followed by electrodialysis (left) and by treatment with ion-exchange resins (right). Left: ———— , R5-F3; —— —— , R8-F2B; - ———- , R8-F1. Right: ———— , C-110; —— —— , C-165; - ———- , C-167; - - - - - , C-168.

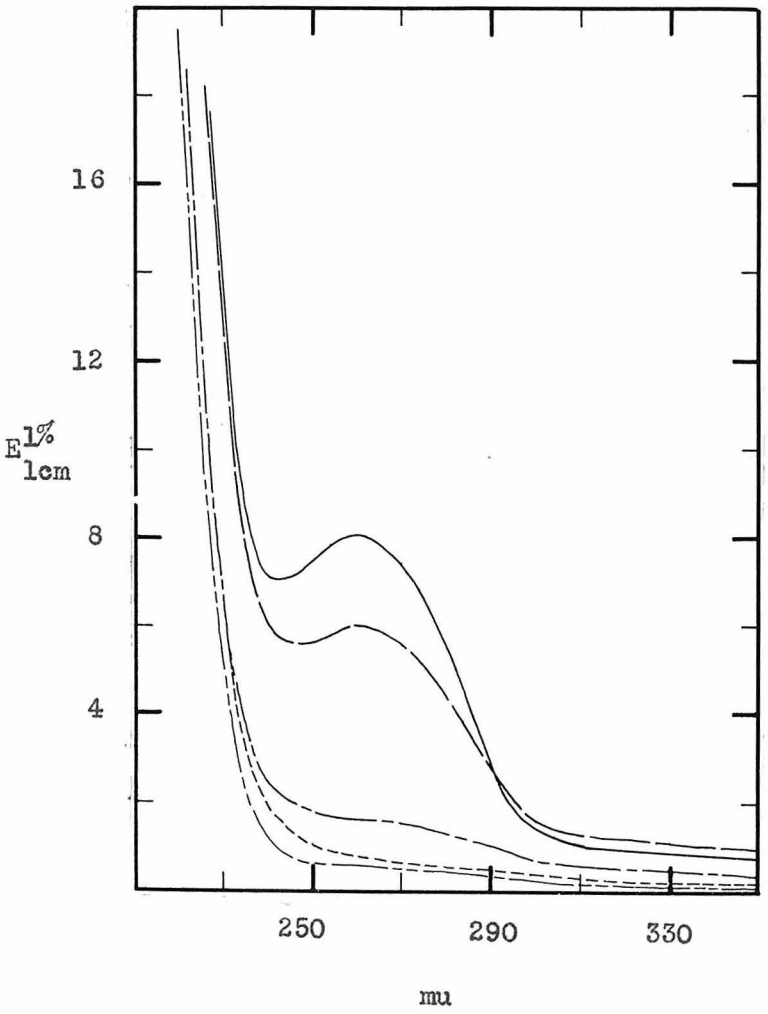


Fig. 6 . Extinction curves of fractions isolated by degradative procedures from hog gastric mucin and from partially purified fractions from mucin (R4-F3 and R5-F3): _____, R5-F3; — —, R4-F3; _ _ _ _, R7-F5A; - - - - - - -, C-47; - - - - -, R6-F5A.

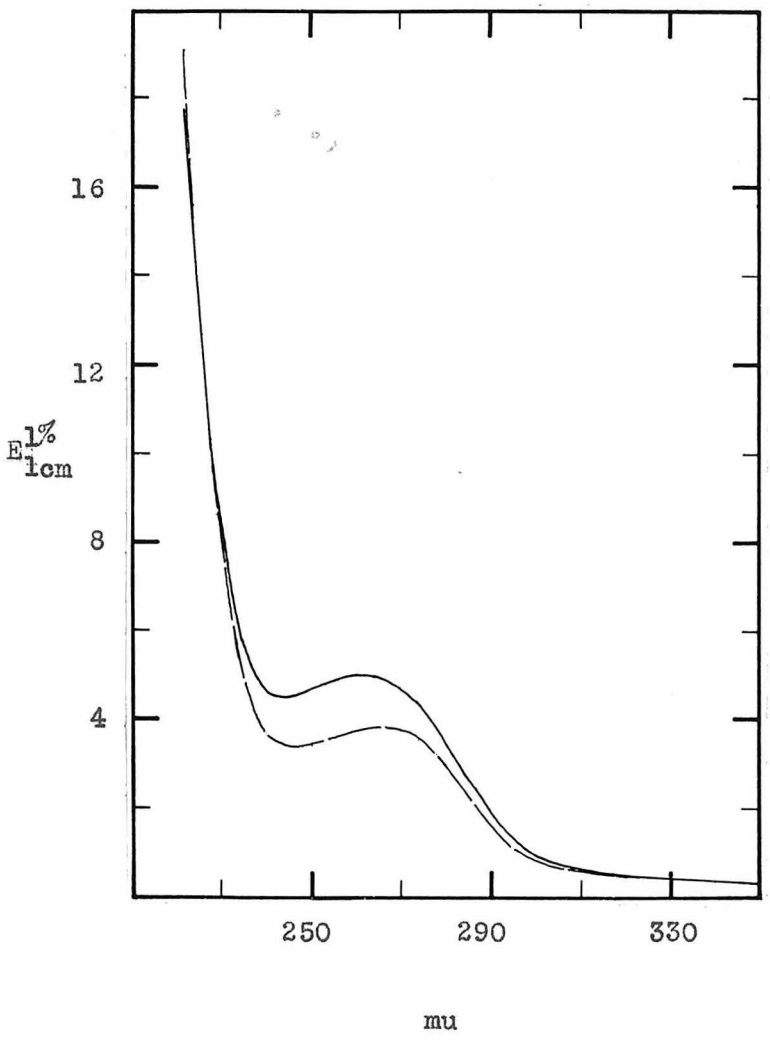


Fig. 7 . Extinction curves of fractions isolated from Wilson pepsin: ———, C-22; — —, C-26.

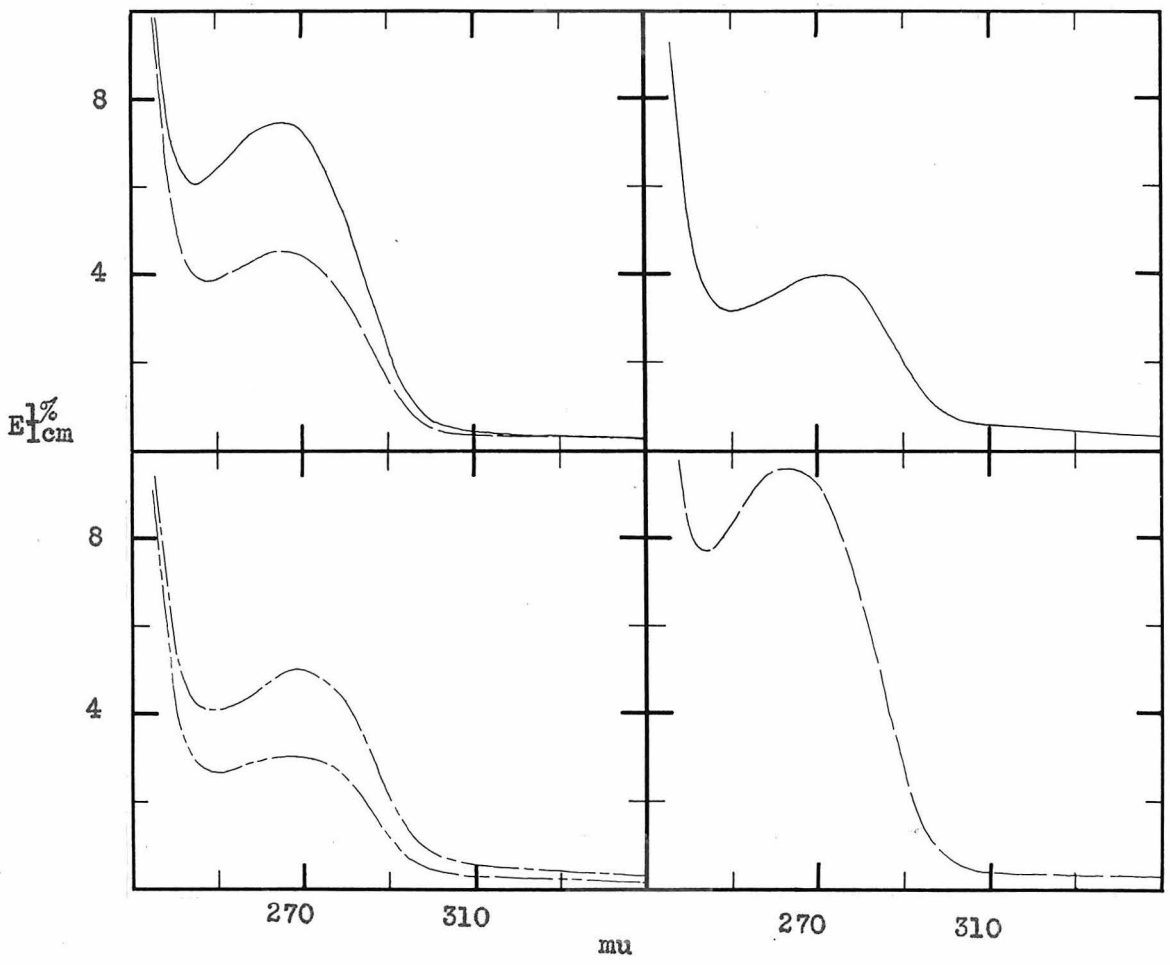


Fig. 8 . Extinction curves of fractions isolated from individual hog stomach linings by alcohol fractionation. Left: ———, H3-F1; — — —, H3-F2; - — — -, H2-F1; - - - - - -, H2-F3. Right: ———, H1-F3; — — —, H4-F1.

IV A Study of the Isolation of Blood Group A Substance from Hog Gastric Mucin by Means of Alcohol Fractionation and Electrodialysis

In Part II the results of a survey of procedures for the isolation of blood group A substance from hog gastric mucin were discussed. On the basis of serological tests it was concluded that all the procedures yielding unmodified preparations (19, 24) gave products of about the same activity. It was further found that these preparations could be shown to be heterogeneous by electrodialysis experiments; two materials, one soluble and one insoluble on electrodialysis were obtained, the former appearing more potent. It seemed probable that with the detailed knowledge of procedures derived from the above survey and with the application of more criteria of fractionation, a more homogeneous preparation could be isolated. In this section the results of such an investigation are reported.

The alcohol fractionation procedure of Landsteiner and Harte (19) with the modifications discussed in Part II was used for preliminary purification. This convenient procedure provides preparations of similar yields and potencies as the sodium sulfate or phenol-alcohol procedures of Morgan and King (24). Electrodialysis in combination with more precise alcohol fractionation was used for subsequent purification. In order to judge the degree of purification achieved the isolation of all fractions was made as quantitative as possible and inactive fractions were partially characterized. Criteria of fractionation involved not only the usual serological tests of inhibition of isoagglutination and hemolysis but also the distribution of total nitrogen, amino nitrogen, and amino acid nitrogen in fractions, analyses for arginine, histidine, and N-acetyl glucosamine, and spectrophotometric analyses. By this program a preparation which

possesses about twice the serological potency of those isolated by other procedures has been obtained. The preparation is not homogeneous, however, and a further study of its fractionation is reserved for discussion in Part V.

Experimental

Serological tests. The inhibitionof isoagglutination (15) and the inhibition of hemolysis (4) tests were carried out as previously described.

Absorption spectra. The spectra were measured as discussed in Part III using solutions at pH 7.20 ± 0.05 and compound concentrations of about 900 micrograms/ml.

Analytical methods. Total nitrogen, amino nitrogen, and amino acid nitrogen were determined in duplicate by means of the Van Slyke manometric apparatus[1]. Additional total nitrogen analyses by the Dumas method and carbon, hydrogen, and sulfur determinations were performed by Dr. G. Oppenheimer and staff. Phosphorus was determined gravimetrically after sulfuric acid-nitric acid digestion as ammonium phosphomolybdate (40).

Arginine and histidine were determined directly on unhydrolyzed solutions of preparations; the Weber modification (43) of the Sakaguchi test was used for arginine and the Pauly diazo test as modified by MacPherson (39) was used for histidine. The latter test

---

[1] I am indebted to Mr. J.P. Cunningham for many of these analyses.

is interfered with by tyrosine (phenylalanine and tryptophan were not found to interfere); the histidine content was calculated by assuming that all the color obtained was due to histidine. No alcohol was added to the reagents in this procedure since it was found that a deep yellow color always appeared; fairly reproducible results were obtained by taking the maximum colorimeter reading observed within two minutes after addition of sodium carbonate. Under these conditions histidine yielded a color which increased rapidly for about two minutes and then slowly thereafter while the color given by tyrosine decreased in intensity immediately after the addition of sodium carbonate. Purified A-substance preparations resembled histidine in color development while impure preparations or inactive fractions, e.g., gastric mucin suspension or R17-F4 (see Fig. 4) resembled tyrosine. Determinations of histidine and arginine were made in duplicate or triplicate on 1 - 5 milligram samples using the Klett-Summerson colorimeter (green filter No. 54). The average percentage deviation of results from the means was about 5 percent for both arginine and histidine.

The equivalent N-acetyl glucosamine content was determined by the method previously described (15).

Acid-base titrations of preparations. About 50 mg of a preparation was dissolved in 5 ml of distilled water. Then 0.1 M HCl was added until the pH was approximately 3; 0.1 ml was usually required. The solutions were titrated with 0.0326 M NaOH with the aid of a Beckman pH meter. Different preparations were compared by recalculating titration curves so that each passed through the same point at pH 8;

this was necessary since aqueous solutions of the preparations differed appreciably in pH. A control consisting of 5 ml of distilled water was titrated similarly.

Electrodialysis apparatus. In the course of the preparative work it became clear that the electrodialysis apparatus in use was much too small for the scale of operations contemplated. The electrodialysis apparatus is shown in Fig. 1; the arrangement without the upper Pyrex pipe represented the usual apparatus and could conveniently handle about 500 ml of solution. Certain aspects of the phenomenon occurring on electrodialysis of A-substance suggested a simple modification which possessed considerably greater capacity without increasing the time of electrodialysis correspondingly. In this modification a four-foot length of standard Pyrex pipe was attached to the center compartment and the pipe jacketed with Pyrex tubing as shown in Fig. 1; the capacity of the middle compartment was thus increased to about 2500 ml. Since the electrodialysis arrangement is somewhat unconventional, the potential gradient being applied to only a small part of the solution, some aspects of its operation are discussed below.

When a solution was electrodialyzed with the earlier arrangement, it was observed that separation of material was apparent not only by an accumulation of solid at the membrane of the anode compartment but also by the separation of a clear supernatant at the surface level of the solution in the middle compartment. The separation at the surface occurred even when the solution in the center compartment was 5 - 10 cm above the horizontal arm of the T-tube where, presumably,

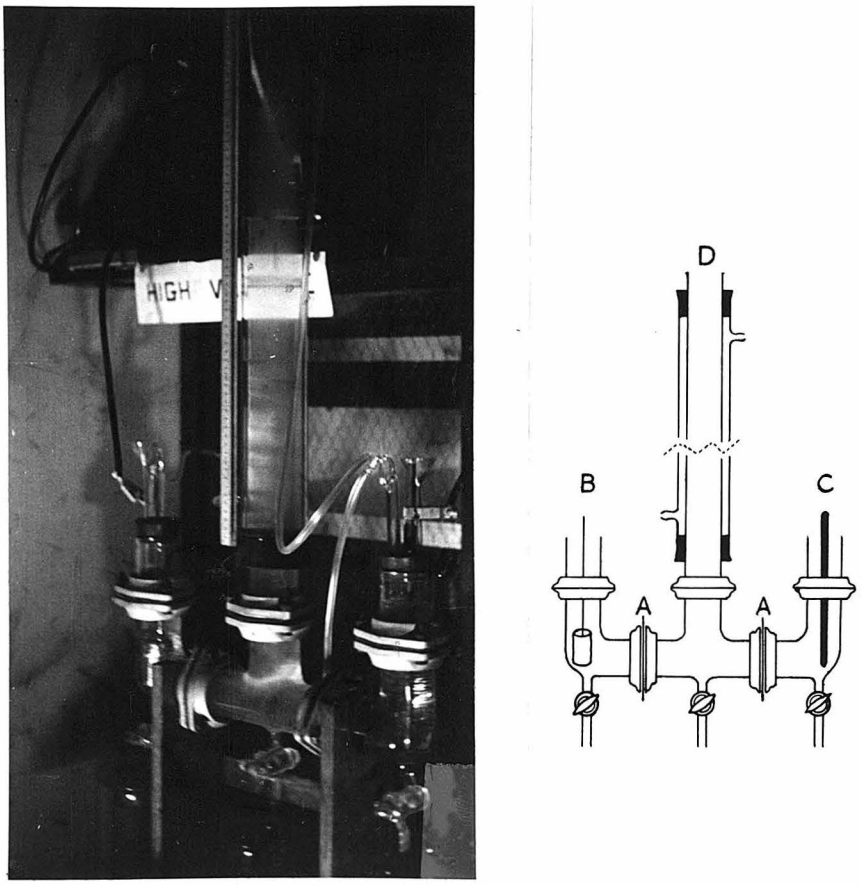


Fig. 1 . Modified electrodialysis apparatus

A Cellophane membrane, two sheets

B Platinum anode

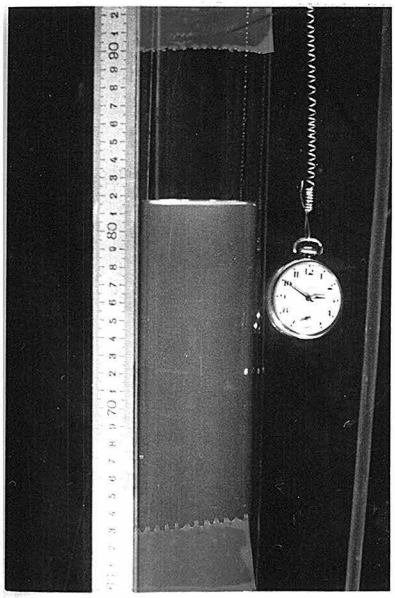
C Carbon cathode

D Central compartment containing solution

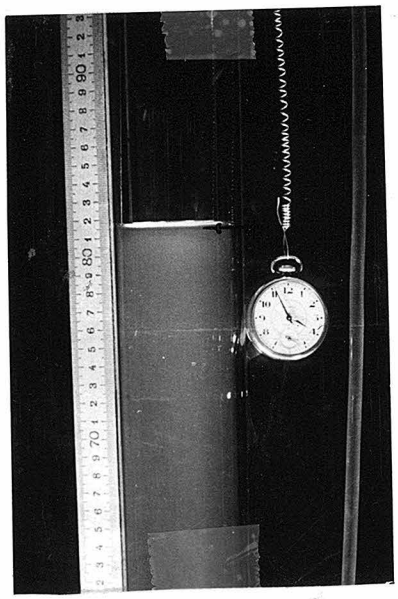
Potential of 700 volts between electrodes controlled by Varitran. Electrode compartments cooled by water passing through glass coils (not shown in diagram).

the potential gradient would be small or negligible. When the electrodialysis was carried out with the modified apparatus, the central pipe being filled to a height of 100 - 120 cm, separation again could be observed at the surface after a lapse of about 45 - 60 minutes. The separation proceeded much more rapidly thereafter. A typical separation at the surface of the solution is illustrated by the photographs shown in Fig. 2. The boundary between the turbid zone and the supernatant was always concave downward when the current was on; when the current was shut off, the boundary slowly settled until it became horizontal and motionless. After applying the current again, the concave surface and the migration of the turbid zone reappeared in a short time.

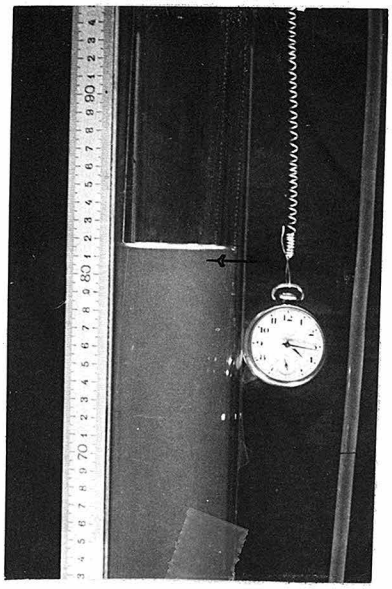
While the separation at the surface was connected with the passage of current, it seemed unlikely that the potential gradient several feet below the surface influenced directly the migration of charged particles at the surface. The rapidity and character of the separation also argued against a simple electrophoretic process. The apparent explanation of the phenomenon was suggested when careful illumination of the central column during electrodialysis revealed the presence of circulation currents. The currents occurred below the boundary between the turbid zone and the supernatant; the supernatant was generally quiescent. Currents on the cathode side of the column were upward and on the anode side of the column, downward. In one instance the rate of mass movement of the liquid upward and downward was found to be, respectively, 22 cm per minute and 10 cm per minute. The liquid moving upward was fairly clear and appeared simply to



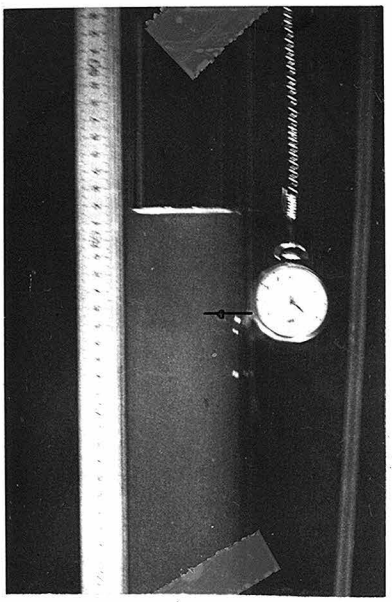
(1)
Electrodialysis begun



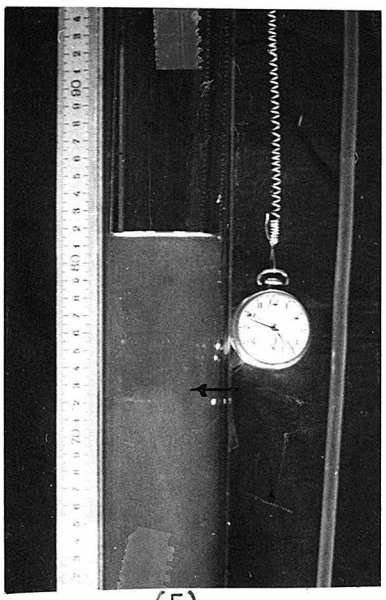
(2)
65 minutes
0,5 cm migration



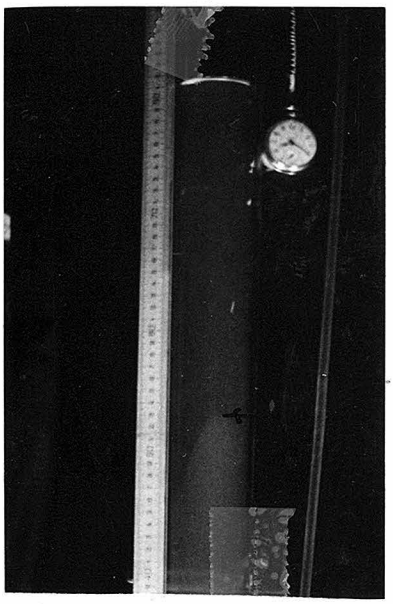
(3)
85 minutes
1,5 cm migration



(4)
100 minutes
5 cm migration



(5)
120 minutes
8 cm migration



(6)
330 minutes
30 cm migration

Fig. 2 . Migration of turbid zone on electrodialysis of A-substance from hog gastric mucin (R18-F7) at pH 7.

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 circulation of liquid and the layering of clear solution coming from the cathode side of the middle compartment above the turbid zone boundary would explain some of the puzzling features of the electrodialysis - the lag in separation, during which the circulation currents are presumably established, followed by accelerated migration of the turbid zone; the shape of the boundary; and the dependence of continued migration on the passage of current. No explanation is available for the origin of the circulation current although migration and precipitation of non-dialyzable, negatively-charged material at the anode membrane, and the resulting pH changes in solution are probably important. The separations occurred more readily in neutral solution than in acid solution; in fact, a completely electrodialyzed solution, whose soluble and insoluble components are mixed giving a solution of pH 3 - 4, would not separate insoluble material again on further electrodialysis. The maintenance of the boundary is perhaps also associated with pH changes and the precipitation of material since the electrodialysis of R18-F7 (see Fig. 2, 8) at pH 7 gave rise to a residue, turbid zone, and clear supernatant, having pH of 3.2, 4.4 and 8.5 respectively. The pH changes occurring on electrodialysis have been noted previously (4).

It was found that occasional turbulence of the boundary during electrodialysis could be minimized by protecting the column from uneven external heating by means of a water jacket.

Results and Discussion

1. The Procedure of Fractionation and the Blood Group A Properties of Preparations

A schematic diagram[1] of the essential operations involved in the separation of A-substance by alcohol fractionation and electrodialysis is shown in Fig. 3. The procedure consisted, successively, of two alcohol fractionations, an electrodialysis, a third alcohol fractionation, and a final electrodialysis and precipitation. About 88 - 90% of the original hog gastric mucin[2] was accounted for and a similar recovery of blood group A activity in mucin is represented by the fractions isolated. The activities of the fractions, determined by isoagglutination inhibition and hemolysis inhibition tests, were calculated relative to hog gastric mucin assumed to have unit activity. The results of the two serological tests were always concordant within the limits of experimental error, the hemolysis test having a precision of 10 - 15% and the isoagglutination test of 50 - 100%.

A characteristic feature of the fractionation is that the A activity is not confined predominantly to a single fraction, a

---

[1] Alcohol concentrations are calculated as volume percent. Solutions were allowed to stand in the cold room (5°) between operations; dialysis of preparations were also carried out in the cold room, usually for at least 5 days, against frequent changes of distilled water. The yield and activity of R17-F1, the inactive water-insoluble fraction from mucin, which was not reserved, was estimated from results obtained in a number of similar preparations.

[2] This estimate includes the 5 - 6% moisture present in Wilson mucin, Item No. 443.

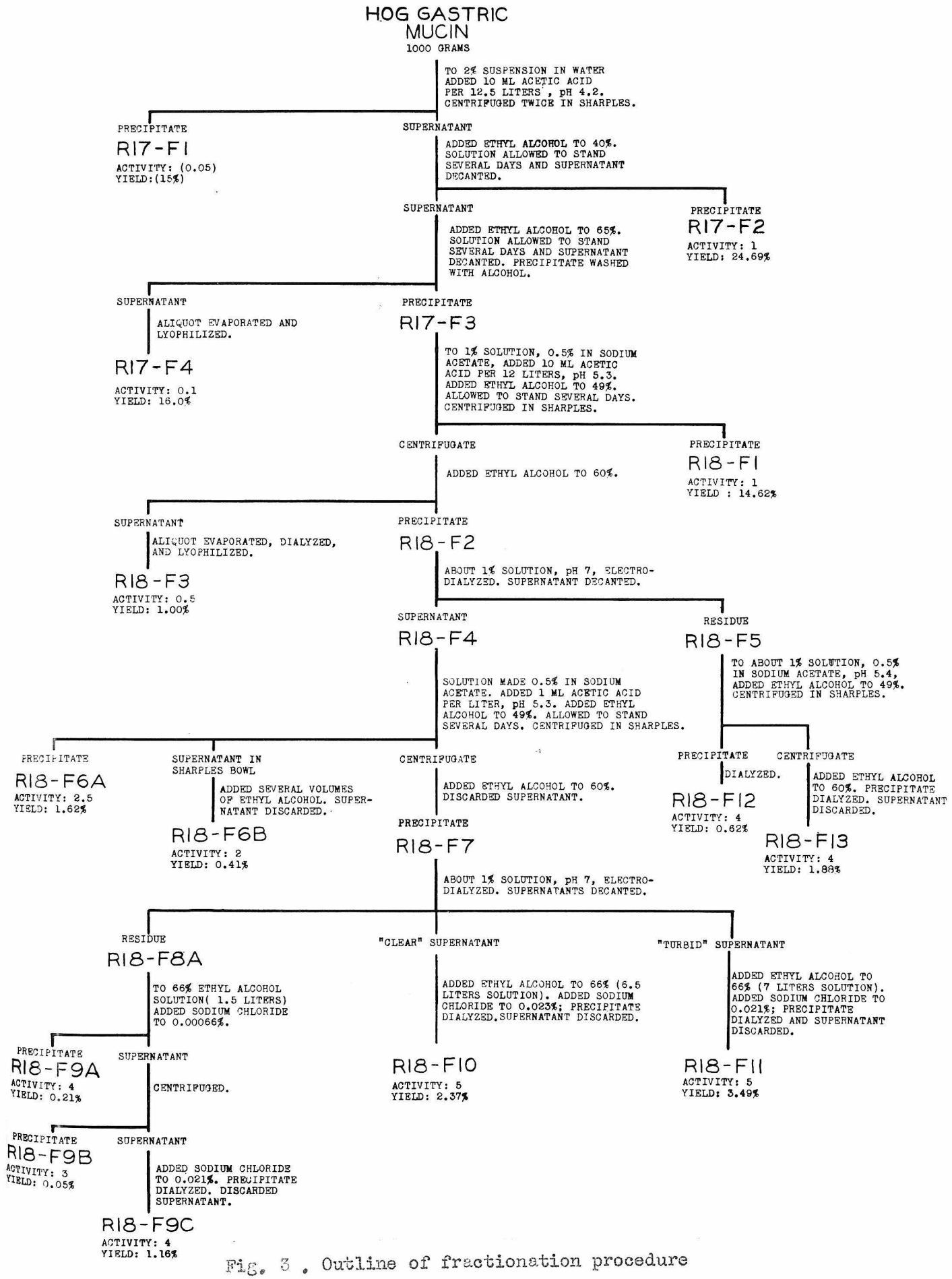


Fig. 3 . Outline of fractionation procedure

result which no doubt reflects in part the complexity of materials in hog mucin and their possible interaction; the presence of a variety of mucoids (25) including the similar O-substance (1, 3), nucleic acid (2), proteinaceous material (24), and amino acids (see Part III) has been reported. In addition the possibility is not excluded that A-substance in mucin may be such a heterogeneous nature that the distribution of activity is unavoidable.

The most active fractions, R18-F10 and R18-F11, were obtained in a total yield of about 5.9% from mucin. The results in Table I demonstrate that the fractions have about twice the activity of those obtained by procedures yielding unmodified preparations (4, 19, 24). Since R18-F10 and R18-F11 possess potencies in the isoagglutination test commensurate with those in the hemolysis test it is concluded that the preparations are undegraded. It is known that degraded preparations on the other hand always show low isoagglutination potencies (see Table I). Furthermore, R18-F10 and R18-F11 would appear to be 25 - 50% more active than preparations previously obtained by a combination of procedures (see Part II). The two fractions were soluble materials obtained on electrodialysis; in agreement with other observations (4), the residues on electrodialysis appeared less active, e.g., R18-F5 and R18-F8A after fractionation with alcohol could not be made to yield material as active as R18-F10 or R18-F11.

Despite the extensive fractionation leading to fractions R18-F10 and R18-F11, evidence discussed in detail later demonstrates the heterogeneity of these preparations. In this connection attention

may be called to the fact that Landsteiner and Harte (19) found that the less active products obtained by alcohol fractionation (corresponds to C-143, Table I) and by treatment with formamide (corresponds to R7-F5A) were homogeneous in the ultracentrifuge. Morgan and King (24) observed that their products were at least 90% homogeneous electrophoretically. These tests are apparently unreliable as criteria of homogeneity since Bendich, Kabat, and Bezer (3) observed that a preparation obtained from mucin by the phenol-alcohol procedure of Morgan and King was only about 60% homogeneous based on a quantitative precipitation reaction with human anti-hog A-substance immune sera (34). This result is in agreement with the observations in this part and previously (Part II) that unmodified preparations with enhanced potencies are obtainable by a variety of methods. However, it seems likely that the authors were incorrect in assuming that inactive contaminant was simply a similar mucoid, presumably O-substance, since it was shown in Part III that other contaminants were possible and also because it is demonstrated below that A and O substances in mucin are separable.

2. Chemical and Physical Properties of A-substance

The properties of the most active preparations, R18-F10 and R18-F11, are summarized in Table II. Some of these properties are discussed in appropriate sections below. The preparations give tests for arginine, histidine and N-acetyl glucosamine; the presence of hexose is indicated by the carbazole test of Gurin and Hood (33). While the elementary analyses are similar to those obtained on A-

Table I

Serological Properties of A-substance Preparations

Reference	Source material	Procedure	Relative A activity[1]	
			Hemolysis test	Isoagglutination test
Landsteiner and Chase 1936 (20)	Pepsin	Alcohol fractionation (C-22)	* 5.5	2.5
Meyer, Smyth, and Palmer 1937 (25)	Hog gastric mucin	Selective adsorption and precipitation(C-47)	* 4.5	1
Landsteiner and Harte 1940 (19)	Hog gastric mucin	Alcohol fractionation (C-143)	2.5	2.5
	Hog gastric mucin	Formamide treatment (R7-F5A)	* 5.5	.5
	Hog gastric mucin	Papain-HCN treatment (R6-F5A)	* 3	1
Morgan and King 1943 (24)	Hog gastric mucin	Sodium sulfate fractionation (R2-F2A)	2.5	1.5
	Hog gastric mucin	Phenol-alcohol fractionation (C-52)	2	1.5
This work	Hog gastric mucin	Alcohol fractionation and electrodialysis (R18-F10 and R18-F11)	5	5

1 Hog gastric mucin, Wilson No. 443, was assumed to have unit activity.

* Procedures presumably involving degradation of active material.

Table II

Chemical and Physical Properties of A-substance Preparations from Hog Gastric Mucin

	Preparation R18-F10	Preparation R18-F11
Total carbon, %	41.6	43.4
Total hydrogen, %	7.3	7.3
Total nitrogen (Dumas), %	5.0	5.2
Total sulfur, %	< .2	< .2
Total phosphorus, %	.04	.04
Ash (residue on Dumas combustion), %	.6	.5
Amino nitrogen, %	.1	.1
Equivalent arginine content, % (before hydrolysis)	.29	.29
Equivalent histidine content, % (before hydrolysis)	.56	.53
Equivalent N-acetyl glucosamine, %	13.1	12.9
Maximum tyrosine content, % (from spectral absorption)	.7	1.0
Maximum tryptophan content, % (from spectral absorption)	.2	.3
Base-combining capacity from pH 3 to 8, moles x 10^5 per gram preparation	8	9
Acid-combining capacity from pH 8 to 10.5, moles x 10^5 per gram preparation	8	8

Table II-(concluded)

	Preparation R18-F10	Preparation R18-F11
Optical rotation (c 1%, phosphate buffer pH 8.0, μ 0.136), α_D^{24}	+7 ± 2	+3 ± 2
Extinction coefficients (citrate-phosphate buffer pH 7.2), $E_{1cm}^{1\%}$ at 260mu	0.91	1.20
at 350mu	.15	.18
Viscosity increment[1], ν_o	225	
Electrophoretic mobility (acetate buffer pH 3.85, μ 0.132, 1.35°C), $cm^2 volt^{-1} sec^{-1} \times 10^5$, anodic	1.0	1.1
Molecular size[2]		
Light scattering	1.7×10^6	
Osmosis	$.26 \times 10^6$	
Diffusion plus viscosity	$0.4\text{-}1 \times 10^6$	

1 Result obtained by Mr. Arthur Pardee (27); ν_o obtained by extrapolation of $\nu = (t/t_o - 1)(1/\phi)$ to zero concentration where t_o and t are times of flow of solvent and solution and ϕ the volume fraction of solute. R18-F10 was found to have a density of 1.52 g/ml in solution.

2 Results obtained by Mr. Arthur Pardee and Mr. Robert Blaker (27).

substance preparations from mucin (2, 3, 19, 24, 25), pepsin (19, 20), and peptone (32), the nitrogen and carbon content appear to be lower by about one percent and two percent respectively. The low phosphorus and sulfur content is in agreement with other observations (19, 20, 24). The high phosphorus of about one percent found by Kabat, Bendich and Bezer (18) for commerical preparations and preparations from mucin would seem to be due to impurities.

The results in Table II indicate the high viscosity and the high molecular weight of A-substance. These properties are treated in detail elsewhere (27).

3. Criteria of Composition Changes on Fractionation

While the specific immunochemical properties of A-substance are responsible for its inherent interest and of primary importance in its detection, these properties are often so difficultly determined quantitatively that they do not afford a precise test of purification in advanced stages of fractionation. The three methods which have been used in assessing serological potency - inhibition of isoagglutination, inhibition of hemolysis, and the quantitative precipitation reaction (18, 34) - have precisions, respectively, of 50%, 10 - 15%, and 12%. An expedient alternative to the improvement of the precision of serological tests, admittedly a difficult problem, is the quantitative assay of a characteristic chemical or physical property of preparations which, in conjunction with serological tests indicating maintenance of potency, serves as a criterion of fractionation. A number of such assays are discussed in this section. In

addition, as the results below demonstrate, the assessment of the properties of all fractions is helpful in revealing small changes in composition of active preparations.

Spectrophotometric analyses. Extinction curves for all the preparations obtained in the course of fractionation are given in Figs. 4 - 11 and extinction values at 260 mu and 350 mu are summarized in Table III. Spectrophotometric analysis has proved to be the most sensitive method so far for following changes in composition. Fractions R18-F10 and R18-F11 (Fig. 8), the two most active fractions obtained, are, in fact, distinguishable only by this method, the latter fraction having somewhat greater absorption.

The distribution of the "260 mu component" of hog gastric mucin (C-135, Fig. 4) in the various fractions is similar to results previously obtained (Part III); the 260 mu component occurs predominantly in the fraction insoluble in 40% alcohol. However, in fractionating the active material insoluble in 60% alcohol (R17-F3, Fig. 5) the 260 mu component is concentrated in the fraction soluble in 60% alcohol (R18-F3, Fig. 5). The extinction of this fraction at 260 mu is the greatest found so far and would indicate that this component represents less than 35% of the solid material in the original mucin suspension.

In agreement with previous observations (Part III), the amino acid-rich material soluble in 60% alcohol on the first alcohol fractionation (R17-F4, Fig. 4) possesses a maximum at 270 mu.

Figs. 4 - 10 show that each step of the procedure involved the separation of fractions which differ appreciably in spectral absorption

Table III

Extinction Values of Fractions Obtained on Alcohol Fractionation and Electrodialysis of Hog Gastric Mucin

Step	Fraction	$E^{1\%}_{1cm}$ 260 mu	$E^{1\%}_{1cm}$ 350 mu
Centrifuged mucin suspension	C-135	22.8	0.9
First alcohol fractionation	R17-F2	36.4	1.79
	* R17-F3	9.63	.77
	R17-F4	36.4	3.73
Second alcohol fractionation	R18-F1A[1]	8.03	.99
	R18-F1B[1]	9.70	1.22
	R18-F1C[1]	8.92	.84
	* R18-F2	3.50	.24
	R18-F3	68.1	4.20
First electrodialysis	* R18-F4	2.67	.26
	R18-F5	5.20	.33
Third alcohol fractionation	R18-F6A	5.05	1.04
	R18-F6B	2.47	.42
	* R18-F7	1.79	.20
Second electrodialysis	R18-F8A	2.52	.25
	* R18-F10	.91	.15
	* R18-F11	1.20	.18
Alcohol fractionation of R18-F5	R18-F12	3.80	.55
	R18-F13	2.15	.18
Alcohol fractionation of R18-F8A	R18-F9A	1.36	.26
	R18-F9B	3.02	.43
	R18-F9C	1.97	.20

* Designates the more active fraction of each step.

1 R18-F1A, R18-F1B, and R18-F1C represent fractions corresponding to R18-F1 (see Fig. 3) obtained in different batches of the preparation.

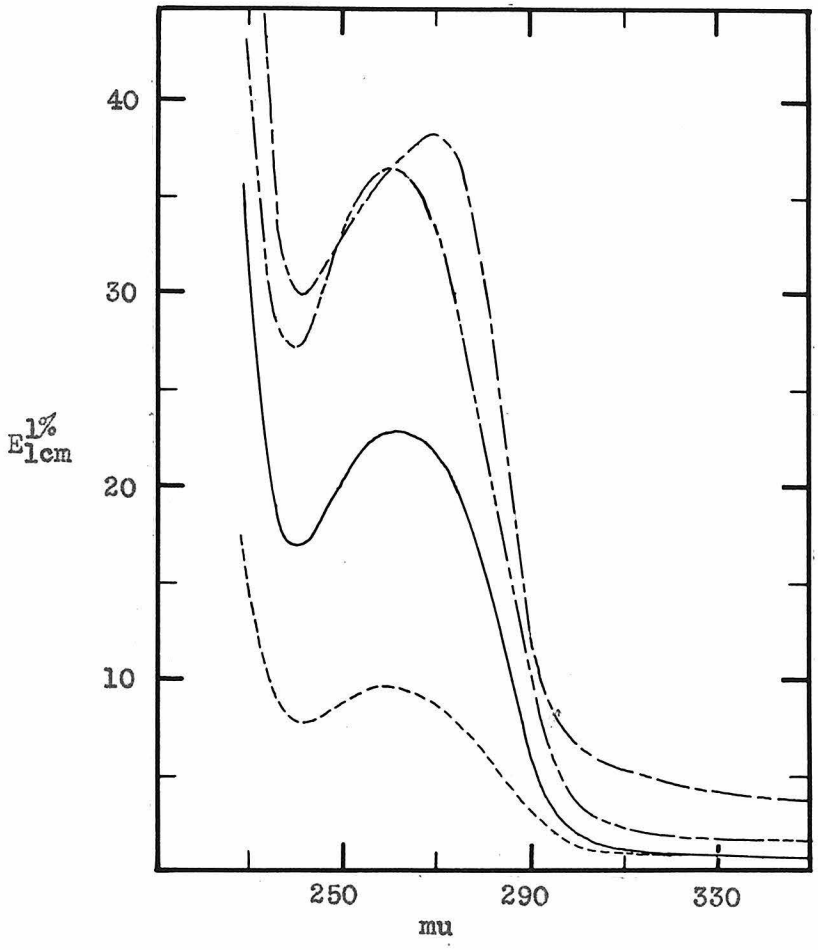


Fig. 4 . Extinction curves of fractions isolated by alcohol fractionation of centrifuged mucin suspension, C-135:-———-, R17-F4;-———--, R17-F2;———, C-135; -------, R17-F3.

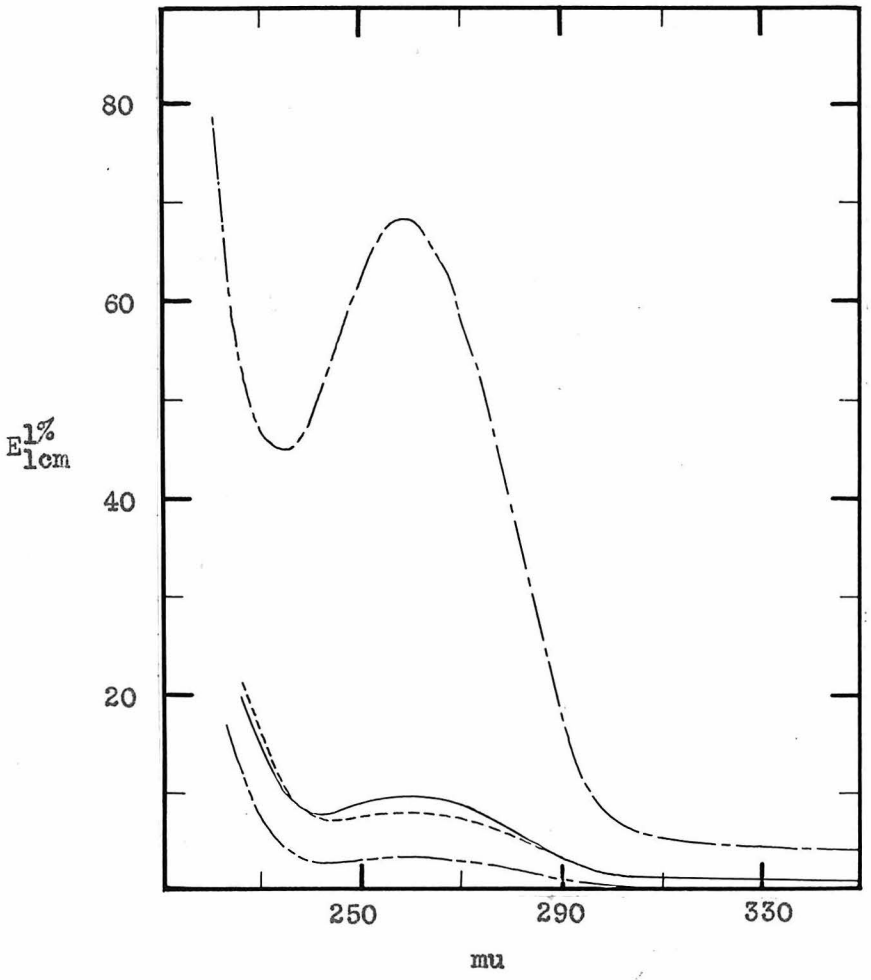


Fig. 5 . Extinction curves of fractions isolated by alcohol fractionation of R17-F3:- ——— -, R18-F3;————, R17-F3; ------, R18-F1A;--------, R18-F2.

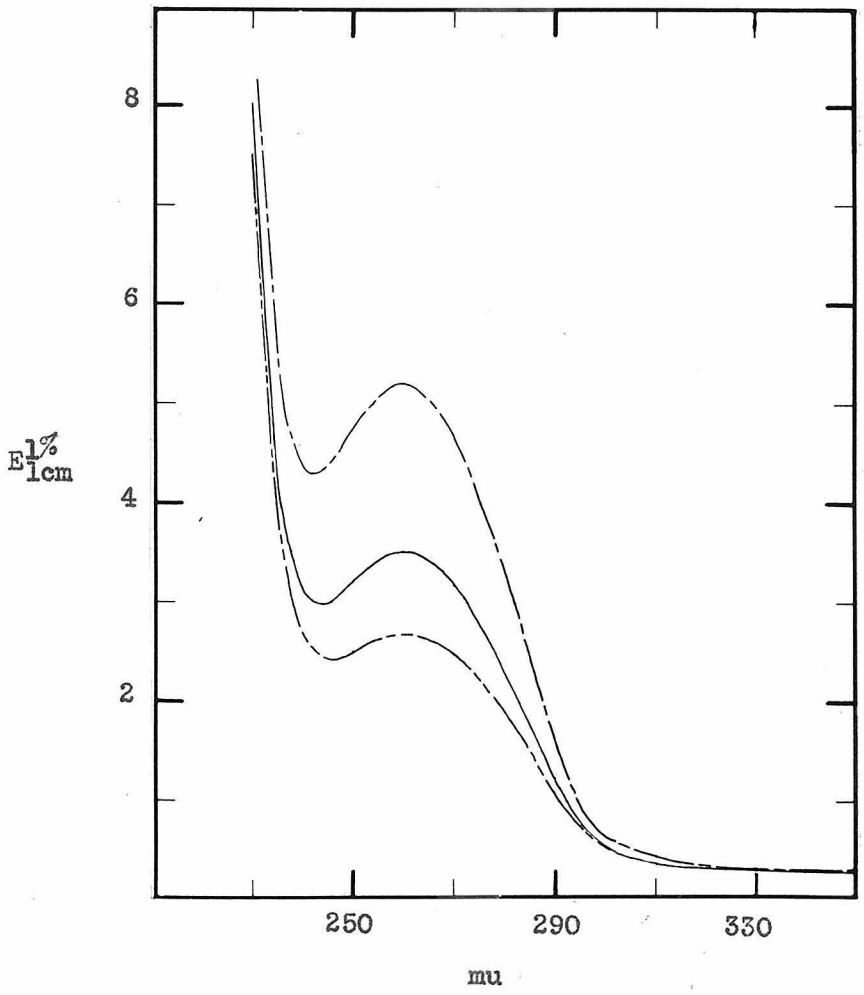


Fig. 6 . Extinction curves of fractions isolated by electrodialysis of R18-F2:- ———, R18-F5; ———, R18-F2; - - - - - -, R18-F4.

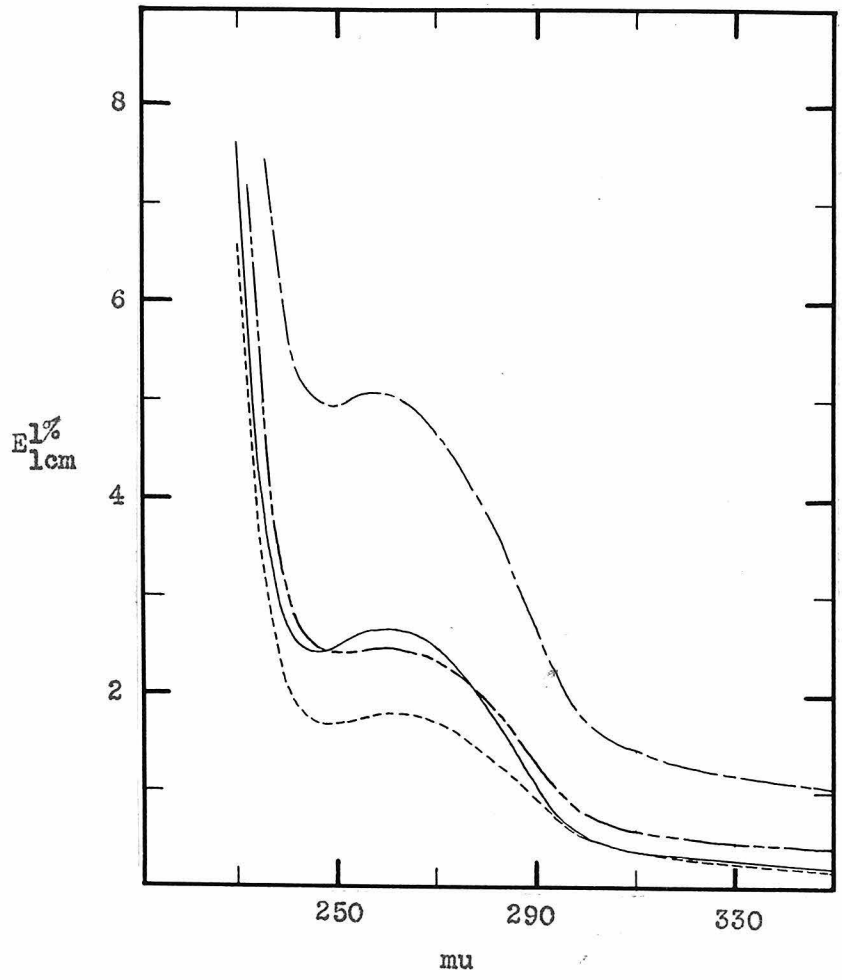


Fig. 7 . Extinction curves of fractions isolated by alcohol fractionation of R18-F4:———, R18-F6A;———, R18-F4;———, R18-F6B;-------, R18-F7.

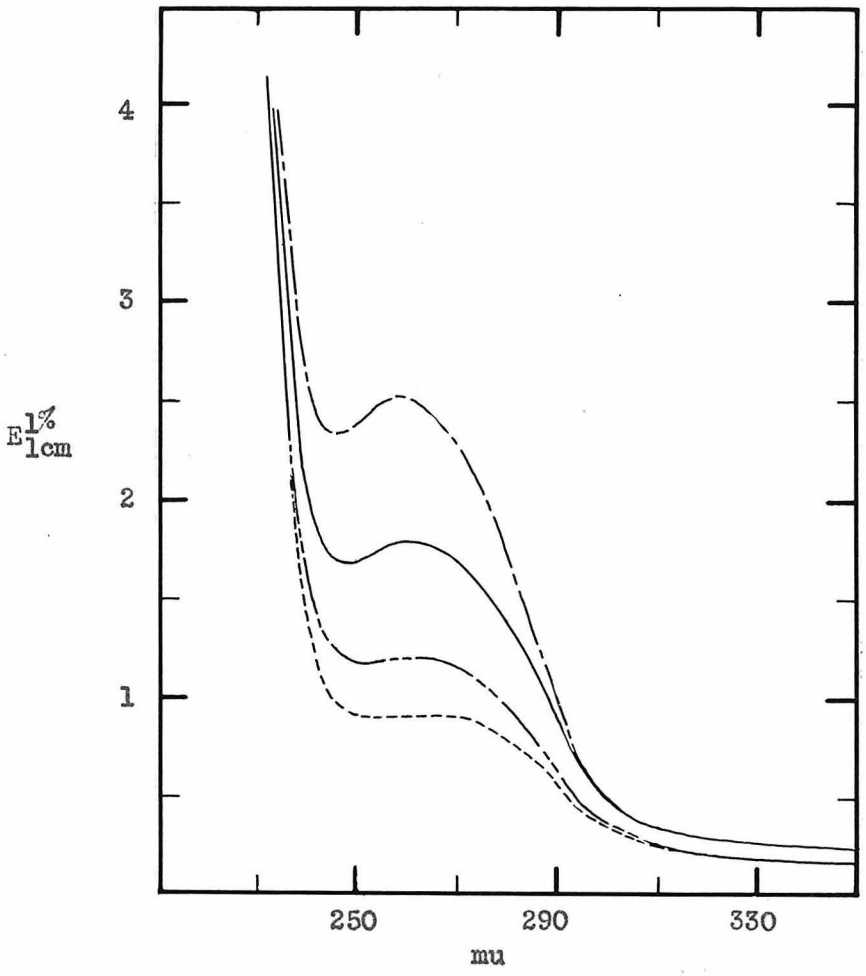


Fig. 8 . Extinction curves of fractions isolated by electrodialysis of R18-F7:– – – –, R18-F8A;————, R18-F7;– – – – –, R18-F11;– – – – –, R18-F10.

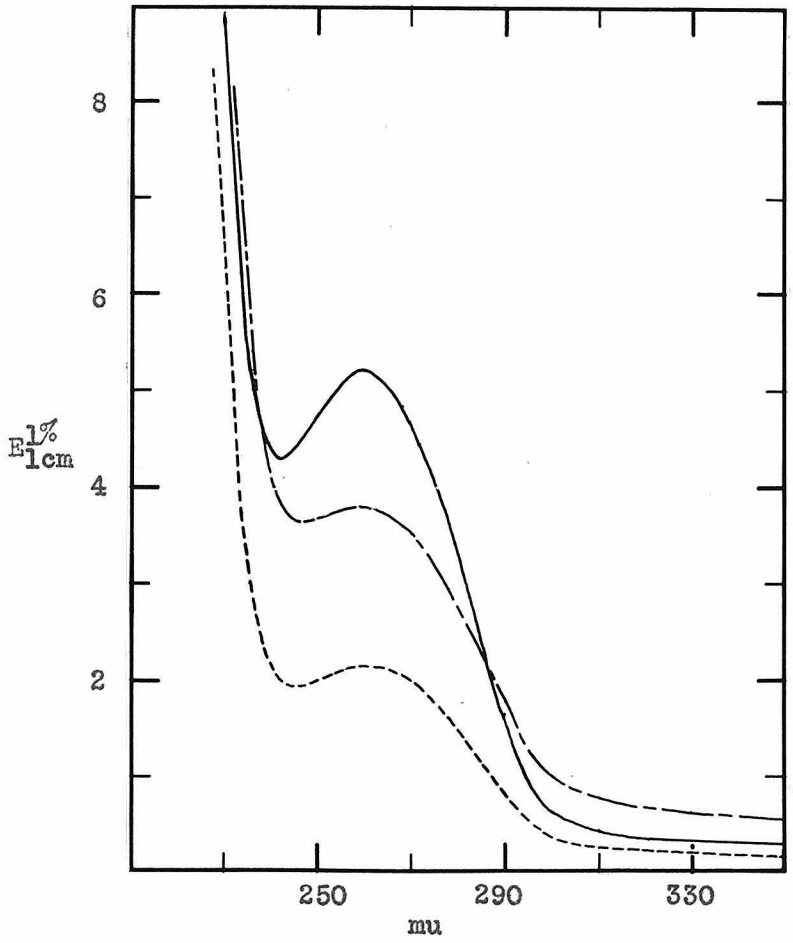


Fig. 9 . Extinction curves of fractions isolated by alcohol fractionation of residue, R18-F5, obtained on electrodialysis of R18-F2:————, R18-F5;— — —, R18-F12;-------, R18-F13.

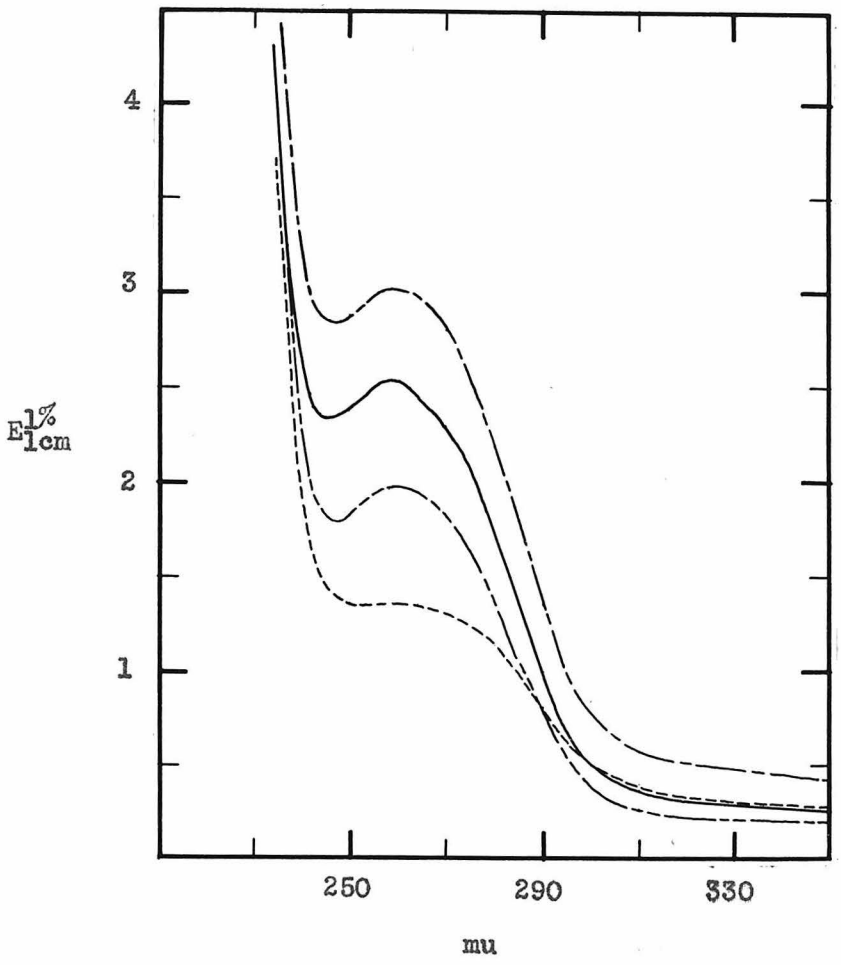


Fig. 10 . Extinction curves of fractions isolated by alcohol fractionation of residue, R18-F8A, obtained by electrodialysis of R18-F7:———, R18-F9B;———, R18-F8A;– – ———, R18-F9C;– – – – – –, R18-F9A.

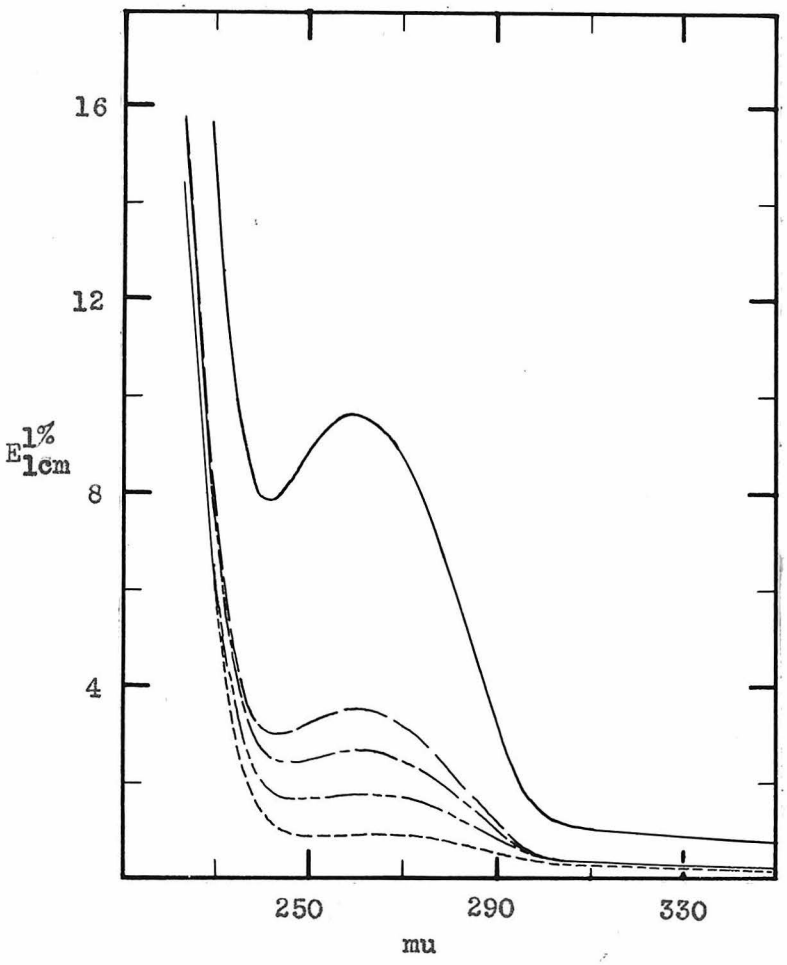


Fig. 11 . Extinction curves of principal fractions obtained on alcohol fractionation and electrodialysis of hog gastric mucin: ——— , first alcohol fractionation (R17-F3); —— ——, second alcohol fractionation (R18-F2); - ——— -, first electrodialysis (R18-F4); - - - - - - -, third alcohol fractionation (R18-F7); - - - - - - -, second electrodialysis (R18-F10).

in the ultraviolet. The more potent fractions show decreasing absorption as may be seen from Fig. 11 which represents the fractions obtained successively in the various steps and leading to one of the most active fractions, R18-F10. The extinction coefficient for this fraction at 260 mu is 30 - 50% smaller than the coefficients for other unmodified preparations isolated from hog gastric mucin (see Table II, Part III). This result confirms the observations in Part III on the low absorption in the ultraviolet of A-substance. The maximum tyrosine and tryptophan content of R18-F10 and R18-F11 were calculated from the extinction coefficients at 280 mu by the method discussed in Part III and the results are shown in Table IV. Assuming a molecular

Table IV

Calculated Maximum Tyrosine and Tryptophan Content of A-substance Preparations

Fraction	$E^{1\%}_{1cm}$ 280 mu	Correction for non-specific absorption, 280 mu	$E^{1\%}_{1cm}$ corrected	Maximum % Tyrosine	Maximum % Tryptophan
R18-F10	0.80	0.30	0.50	0.7	.2
R18-F11	.97	.27	.70	1.0	.3

weight of 250,000 for A-substance (27), the maximum tyrosine and tryptophan contents indicate the presence of a maximum of about 2 molecules of tryptophan and about 10 molecules of tyrosine per

molecule of A-substance. If both amino acids were present, these estimates would, of course, be too large.

Since considerable differentiation of fractions in the final step of the fractionation procedure is still evident - e.g., compare R18-F8A, the residue, and R18-F10, the supernatant, on electrodialysis (Fig. 8) - it would appear that the possibilities of further fractionation have not been exhausted. In Part V, such fractionation assisted by spectrophotometric analysis is discussed.

Nitrogen analyses. The total nitrogen content of fractions is useful in detecting changes in composition in the early steps of the fractionation procedure. The most active preparations contain about 5% nitrogen; the limiting nitrogen content within experimental error is attained, however, after the first electrodialysis (see Table V). Less active fractions contain more nitrogen with values varying from 5.5 to 10%.

Amino nitrogen and amino acid nitrogen analyses are poor criteria of fractionation after the first step in the procedure. Most of the amino acids occur in the fraction soluble in 60% alcohol, e.g., R17-F4 contains about 25% free amino acids. The amino nitrogen content of many of the preparations is less than 0.1% after the second alcohol fractionation so that it is not possible to use this analysis for detecting small changes in composition.

Equivalent N-acetyl glucosamine content. The correlation of the equivalent N-acetyl glucosamine content, determined with Ehrlich's reagent by the method of Morgan and Elson (36), with the serological properties of fractions has been demonstrated previously (4, 15). A

Table V

Properties of Fractions Obtained on Alcohol Fractionation and Electrodialysis of Hog Gastric Mucin

Step	Fraction	Total N	Amino N	Amino acid N	Equivalent % arginine	Equivalent % histidine	Equivalent % N-acetyl glucosamine
	Gastric mucin	8.5	1.7	1.3	1.6	2.7	
Centrifuged mucin suspension	C-135				1.4	2.0	7.6
First alcohol fractionation	R17-F1	9-10					
	R17-F2	8.9	1.0	1.0	1.4	1.9	7.5
	* R17-F3	7.1	.1		.65	.99	11.6
	R17-R4	10.5	4.6	4.4	4.3	5.3	.9
Second alcohol fractionation	R18-F1A	6.8	.3		.57	.93	9.9
	R18-F1B	7.4	.3		.63	1.07	10.5
	R18-F1C	7.0	.4		.58	.99	10.4
	* R18-F2	5.9	.2		.33	.57	12.5
	R18-F3	9.1	.8		2.0	1.24	4.0
First electrodialysis	* R18-F4	5.0	.1		.35	.58	12.5
	R18-F5	6.0	.1		.32	.55	12.1
Third alcohol fractionation	R18-F6A	6.7	.1		.45	.77	10.9
	R18-F6B	5.5	.2		.35	.72	12.9
	* R18-F7	4.9	.1		.29	.52	12.3
Second electrodialysis	R18-F8A	5.5	.1		.27	.50	12.4
	* R18-F10	5.0	.1		.29	.56	13.1
	* R18-F11	5.2	.1		.29	.53	12.9
Alcohol fractionation of R18-F5	R18-F12	6.2	.1		0.38	.70	12.9
	R18-F13	5.3	.1		.28	.50	12.6
Alcohol fractionation of R18-F8A	R18-F9A	5.5	.1		.31	.62	12.7
	R18-F9B	6.1	.1		.35	.64	12.0
	R18-F9C	5.6	.1		.25	.48	12.0

* Designates the more active fractions of each step.

similar correlation exists for the fractions isolated in this procedure as may be seen from the results in Table V. The most active fractions attain a maximum N-acetyl glucosamine content of about 13% after the first electrodialysis. Less active fractions show less N-acetyl glucosamine.

Since the proof that N-acetyl glucosamine in A-substance is in fact the moiety reacting with Ehrlich's reagent has been indirect (24) and because the rate at which the colors are produced for A-substance and N-acetyl glucosamine are substantially different (15), it was of importance to determine if the chromophoric groupings in the reaction products of p-dimethylaminobenzaldehyde with N-acetyl glucosamine and A-substance differed. Accordingly the absorption spectra of the red colors obtained for N-acetyl glucosamine and A-substance with Ehrlich's reagent were determined; the spectra were essentially identical as may be seen from Fig. 12. Several aspects of the reactions on which Ehrlich's test is based are discussed in detail in Part VII.

Arginine and histidine content. These analyses were carried out without hydrolysis of A-substance and thus do not represent the actual content of the amino acids in the preparations since it is well known (23) that low values are usually obtained in this way. It is to be noted that the histidine test is not specific since tyrosine gives a similar color test. However, the color obtained with tyrosine is only about 25% of the intensity of the color obtained with an equal weight of histidine. Furthermore it is known that the color obtained for arginine in the Sakaguchi test is diminished in the presence of histidine and ammonia (41, 43). These considerations, while of importance

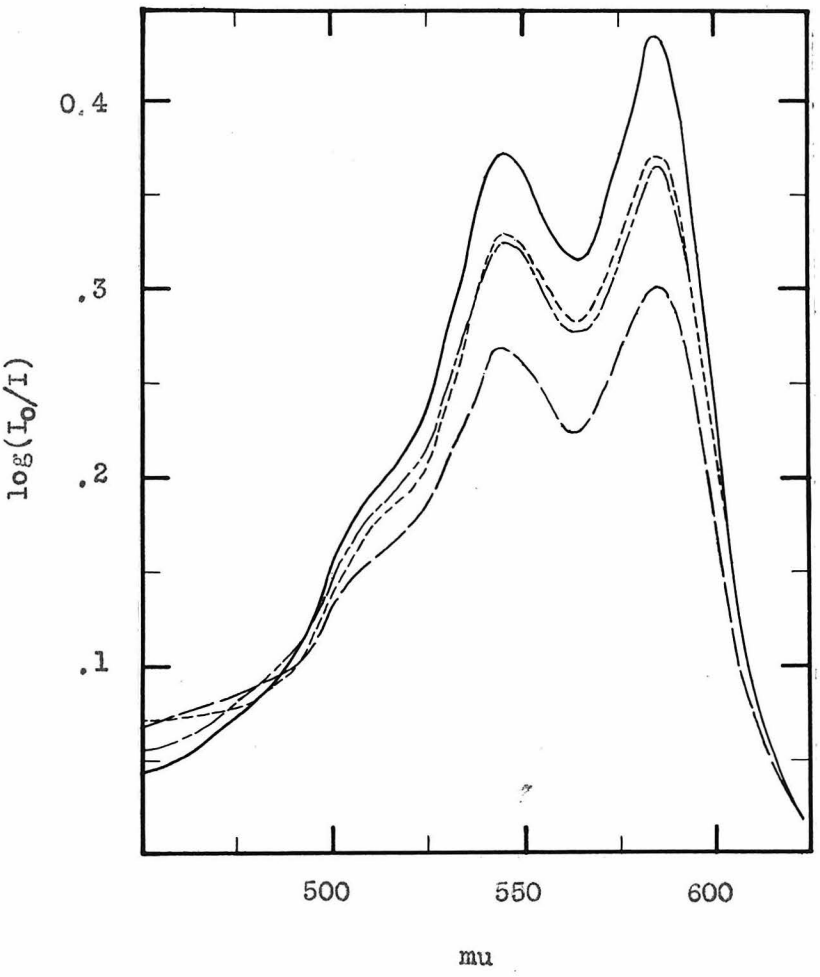


Fig. 12 . Absorption spectra of colors obtained with Ehrlich's reagent for A-substance from hog gastric mucin and for N-acetyl glucosamine:———, 150 γ N-acetyl glucosamine;------, 1000 γ R18-F10;- ——-, 1000 γ R18-F11; ——, 100 γ N-acetyl glucosamine.

in interpreting results in terms of particular functional groups, do not influence the significance of the results in detecting changes in composition.

Table V indicates that a constant content of about 0.3% arginine and 0.5% histidine is found after the first electrodialysis. Less active fractions usually have considerably larger quantities of the amino acids. That the color obtained in the Pauly diazo test was in large part due to histidine is evident since the calculated tyrosine content based on this color test for R18-F10, 2.2%, is considerably larger than the maximum tyrosine content, 0.7%, found by spectrophotometric analysis. Brand and Saidel (6) reported 0.6% histidine in A-substance isolated by phenol-alcohol fractionation from hog gastric mucin. Qualitative tests for histidine as well as arginine have been reported (19, 20).

The persistence of the tests for arginine and histidine on extensive fractionation confirms earlier observations by Landsteiner and Harte (19) and Morgan and King (24) that amino acids constitute an integral portion of the A-substance molecule.

Acid-base titrations. Hog gastric mucin possesses considerable capacity for binding acid; this was presumably of importance for the effective action of mucin in ulcer therapy (31). The presence of acid polysaccharides containing uronic acid, N-acetyl glucosamine, and sulfuric acid was detected by Meyer, Smyth and Palmer (25). It has been shown by Mr. E.L. Bennett of this Laboratory that the fractionation of mucin leading to A-substance produces material having very little buffer capacity. This observation has been confirmed in this

study; the titration curves for R18-F10 and R18-F11 as well as a few less active fractions are shown in Fig. 13. While some differentiation of fractions is observed by this method - e.g., R18-F12 has considerable buffering capacity, - in general, the buffering capacity of fractions are similar in the advanced stages of fractionation (compare R18-F10, R18-F11, R18-F9C, and R18- F13). The buffering capacity, while low, appears to be significant; this result is at variance with the observation of Morgan and King (24) that no groups ionizing between pH 2 and 10.5 were detectable in A-substance from mucin.

It is of interest to attempt to correlate the acid-base properties of A-substance with what is known concerning its acidic and basic constituents. In a titration of A-substance solution from pH 3 to pH 8 one would expect to neutralize various acidic groupings. The absence of phosphorus and sulfur and the low or negligible content of uronic acid (25) eliminates these groups as of importance. So far as is knowns the only other possible groups which might be neutralized are those occurring in amino acids. For example, terminal carboxyl groups in polypeptide chains (pK' 2-3)[1] would be only partially neutralized; side chain carboxyl groups would be estimated (pK' of aspartic acid and glutamic acid, 3.9 and 4.3); and the imino group of histidine (pK' 6.1) would be estimated. The absence of sulfur excludes a contribution from cysteine (pK' 8.2). Assuming the absence of amides and the equal availability of each amino acid towards base, the maximum contribution of histidine, aspartic acid and glutamic acid to

---

[1] pK' is Brönsted constant and values have been taken from reference (42).

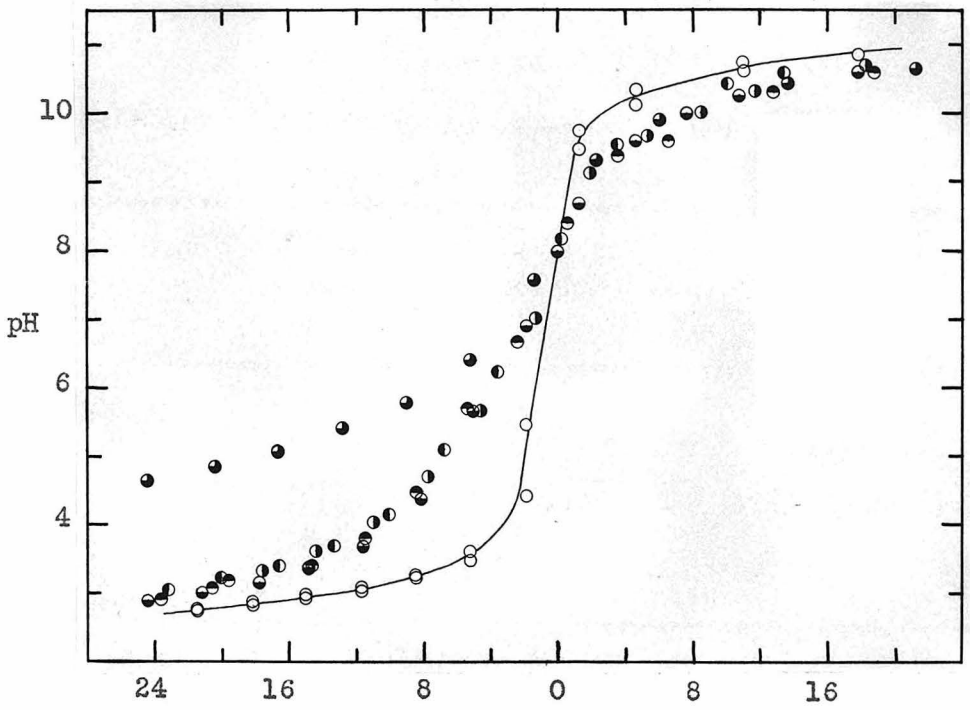


Fig. 13 . Acid-base titration curves of A-substance preparations. —○— , control; ◒ , R18-F10; ◓ , R18-F11; ◑ , R18-F9C; ◐ , R18-F13; ◕ , R18-F12.

base neutralization can be calculated from the data of Brand and Saidel (6) and results are shown in Table VI.

Table VI

Calculated Base-combining Capacity of A-substance

Amino acid	Per cent present	Moles of base x 10^5 per gram of A-substance (calculated)
Histidine	0.6	4
Aspartic acid	.8	6
Glutamic acid	1.3	9
		total 19

This estimate is probably high due to the assumptions made but it is of interest that R18-F10 which is probably more homogeneous than the above preparation, is observed from Fig. 13 to have a base combining capacity of approximately the same magnitude, about 8 x 10^{-5} moles/gram [1]. Similarly, a titration from pH 10.5 to 8 would partially estimate terminal amino groups (pK' 8-9.5), side chain amino groups (lysine, pK' 10.5), and the phenolic group of tyrosine (pK' 10). From Fig. 13 the acid-combining capacity of R18-F10 over the range pH 10.5 - pH 8 is estimated to be about 8 x 10^{-5} moles/gram. The

[1] This value was calculated from the number of moles of hydroxyl ion required to bring the solution containing A-substance from pH 3 to pH 8 minus the number of moles required to titrate a blank solution over the same range. The value is uncertain due to the fact that no attempt was made to apply activity coefficient corrections (42).

lysine and tyrosine content found by Brand and Saidel (6) would suggest a maximum of 10×10^{-5} moles/gram.

It must be emphasized that little quantitative significance is attached to the particular values which were calculated or observed for the acid or base-combining capacity of A-substance because of the assumptions involved; the calculations serve merely to indicate a reasonable order of magnitude for these values.

4. Studies on the Homogeneity of Preparations

Serological specificity. The heterogeneity of R18-F10 and R18-F11, the two most active preparations isolated, was immediately evident as a result of observations on their serological specificity[1]. It has been mentioned previously (Part III) that as a result of the observations of Witebsky (29), Bendich, Kabat, and Bezer (3), and Aminoff, Morgan, and Watkins (1), hog gastric mucin was shown to be an artificial mixture of A and O-substances. Morgan and coworkers also reported that the heterogeneity of mucin could be demonstrated by the relative A and O activity of preparations and mention was made of the fact that by this means a partial separation of A and O-substances in mucin was detectable. The possibility of separation seemed doubtful (3) because of the similar elementary composition and physical properties of A and O-substances. The tests which have been made by Dr. Morgan on our preparations confirm the separation of A and O-substance

---

[1] We are indebted to Dr. W.T.J. Morgan of the Lister Institue of Preventive Medicine, London, England for these observations (private communication, May 2,1947).

as well as demonstrating their heterogeneity (Table VII).

Table VII

Immunological Evidence for the Heterogeneity of A-substance Preparations

Fraction	Agglutination inhibition titer	
	Anti-A[1] x 10^3	Anti-O[2] x 10^3
C-135	1:320	1:1280
C-22	1:640	1:10
R18-F10	1:640	1:320
R18-F11	1:640	1:640
Lister Standard[3]	1:640	1:2560
"O" Standard[3]	-	1:5120

[1] Inhibition of isoagglutination of human blood group A cells by serum of individuals of blood group B.

[2] Inhibition of agglutination of human O cells by selected cattle serum (37, 38).

[3] The Lister standard is an A-substance preparation isolated from hog gastric mucin; O-substance was isolated from a human pseudomucinous ovarian cyst fluid. These preparations are standards used by Dr. Morgan in his A - O titrations.

The Wilson mucin which has been used in our studies possesses O activity; C-135, which represents all the solid in solution after centrifuging off insoluble material from a mucin suspension, has 25% of the O activity of human O-substance and 50% of the activity of Dr. Morgan's A-substance preparation from mucin. R18-F10 and R18-F11,

however, have only 6 - 12% of the O activity of human O-substance or 25 - 50% of the O activity of the starting material, C-135. The A activity of R18-F10 and R18-F11 is twice as great as C-135 which is in agreement with our observations in view of the rather poor precision of the agglutination test carried out by the serial dilution technique. It is not possible at present to estimate quantitatively the contamination of the A-substance by O-substance since the degree of purity of the O-substance used as a standard and the extent to which O-substance from hog stomach resembles human O-substance immunochemically are not known[1].

It is of interest that C-22 which was obtained from Wilson pepsin (4) by alcohol fractionation shows very little O activity. This could be due to the fact that 1) pepsin contains very little O-substance, 2) the fractionation procedure separated A and O substances excellently, or 3) degradation of O-substance and consequent loss of serological activity occurred. The first possibility seems unlikely since pepsin is a pooled source material. One of the latter two alternatives can not be selected since the stability of O-substance is not known. However, degradation seems a more probable explanation since the fractionation procedure for pepsin is not extensive and, by analogy, because A-substance from pepsin appears to be slightly degraded (4).

Electrophoresis. Morgan and King (24) have reported that A-

---

[1] It is to be noted that another uncertainty is possible because the stability of O-substance to various reagents and conditions as measured by immunochemical tests is not known and, despite the mild conditions used in the procedure described here, any degradation of O-substance would be interpreted as an apparent decrease in heterogeneity.

substance from hog gastric mucin is 90% homogeneous in the Tiselius apparatus. Fig. 14 shows that R18-F10 and R18-F11 are also essentially homogeneous by electrophoresis[1]. The mobilities (Table VIII) of these preparations at pH 3.9 are quite low[2] and the migration of boundaries is anodic in agreement with an extensive investigation of of the electrophoretic properties of A-substance from hog gastric mucin made in this Laboratory by Mr. David H. Brown and Mr. Edward L. Bennett. It is to be noted that electrophoresis has not been found

Table VIII

Mobilities of A-substance in Acetate Buffer pH 3.85[3]

Fraction	Mobility, anodic $cm^2 volt^{-1} sec^{-1} \times 10^5$
C-127	0.8
R18-F10	1.0
R18-F11	1.1

[3] μ 0.132 (0.032 M sodium acetate, 0.0173 M acetic acid, 0.100 M sodium chloride), 1.35°C, 0.5% solutions of preparations.

to be a particularly sensitive criterion of homogeneity. Thus in Fig. 14, C-127, which represents all the material precipitable by

1 I am indebted to Mr. David H. Brown for these measurements.

2 The mobilities were calculated from the descending boundaries using the equation of Longsworth and MacInnes (35), $u=A(a-d)(K/it)$.

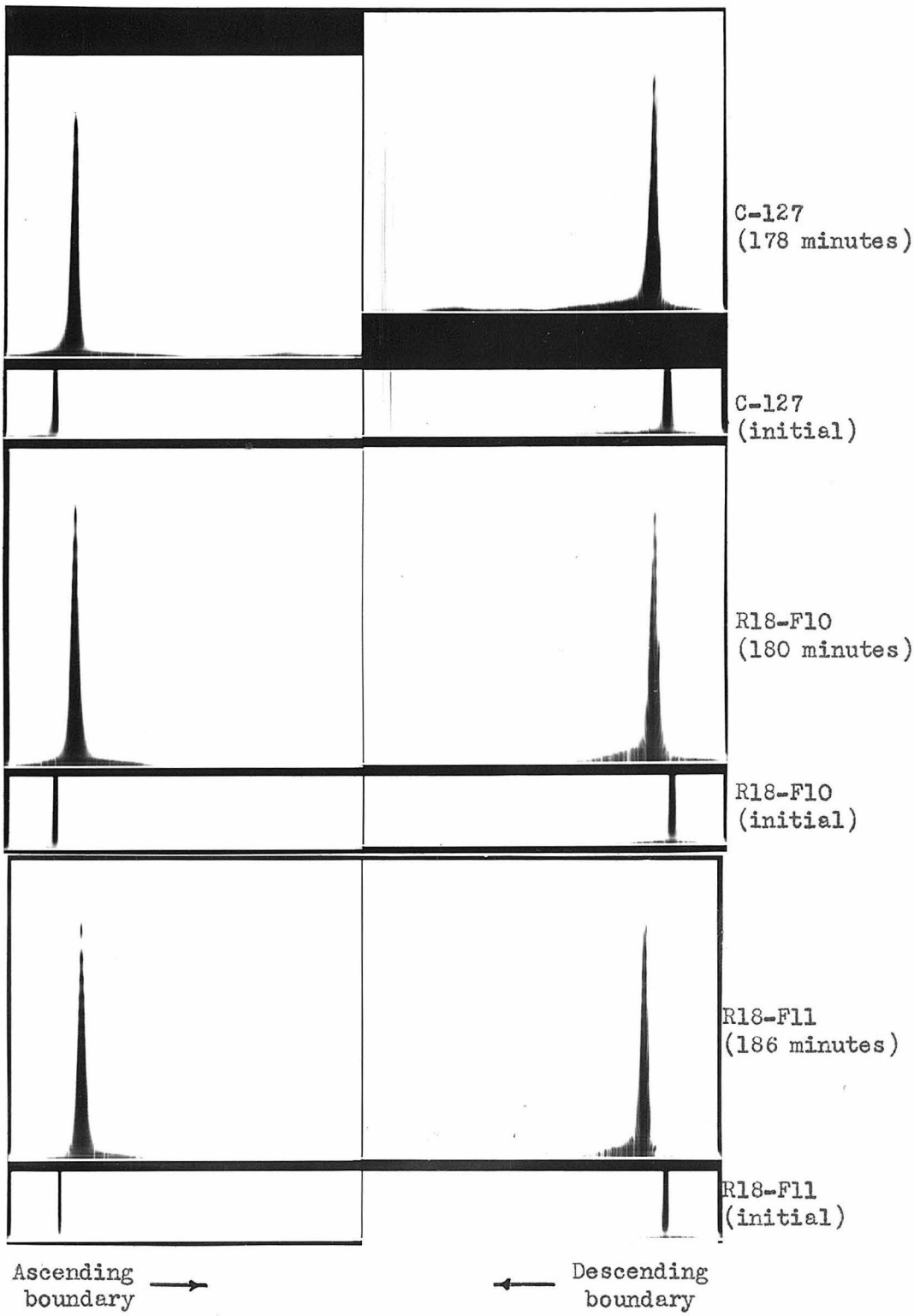


Fig.14 . Electrophoresis diagrams of A-substance preparations. (0.5 % solutions, 1.35°C, acetate buffer pH 3.85, μ 0.132).

60% ethanol from a mucin suspension and having only about one-third the serological potency of R18-F10 and R18-F11, appears to be as homogeneous as these latter preparations (a very small amount of a fast moving component is detectable, however, in C-127).

Molecular size studies. Studies of the molecular size of blood group A-substance from hog gastric mucin have been carried out in this Laboratory by Mr. Arthur Pardee and Mr. Robert Blaker (27). These studies suggest that the active preparations discussed above, R18-F10 and R18-F11, are heterogeneous in respect to molecular size. Light scattering measurements which yield a weight average molecular weight, gave a molecular weight of 1,700,000 for preparation R18-F10 in 0.15 M saline. A number average molecular weight for the same preparation by osmotic pressure measurements in 0.15 M saline plus 0.02 M phosphate buffer, pH 7.1, gave a value of only 260,000. As has been pointed out a few percent of a quite high or low molecular weight material could be responsible for the discrepancy (Mr. Pardee has calculated, for example, that 3% of material of molecular weight 10^4 with 97% molecular weight 10^6 could account for the discrepancy). It is significant that the less active preparation, R18-F2, obtained in the course of isolation is even more heterogeneous by this criterion. This preparation has about the same number average molecular weight as R18-F10 but it possesses a weight average molecular weight by light scattering of more than 3,000,000 in formamide solution.

Summary

By a combination of alcohol fractionation and electrodialysis a purified A-substance preparation has been isolated from hog gastric mucin. The preparation has greater activity in the inhibition of hemolysis and isoagglutination tests than preparations hitherto derived from this source. Many physical and chemical properties for this preparation are reported. The applicability of various tests for following changes in composition on fractionation is discussed.

The preparation isolated is heterogeneous on the basis of serological specificity and molecular size studies and homogeneous by electrophoresis.

V The Occurrence of Acid-soluble and Acid-insoluble Forms of Blood Group A Substance in Hog Gastric Mucin

The fractionation of hog gastric mucin by means of electrodialysis and alcohol precipitation (Part IV) led to the isolation of a highly potent A-substance which was heterogeneous as to serological specificity and molecular size and homogeneous by electrophoresis. Aside from the evidence afforded by the direct tests of homogeneity, the possibility of the further purification of the products seemed likely in as much as significant differentiation in the ultraviolet absorption spectra of fractions was evident in the final step of the isolation procedure. However, the repeated application of the above or other usual methods of fractionation was deferred since a number of phenomena previously observed suggested a new approach to the fractionation of A-substance. As a result it has been possible to separate the potent products obtained by alcohol precipitation and electrodialysis into two fractions differing strikingly in their solubility in aqueous solutions at low pH and ionic strength. Both fractions have potent A activity and the occurrence of the fractions in hog mucin is obviously of importance in interpreting results on the isolation and characterization of A-substance from hog stomach sources. The procedure yielding the two forms involves separation of fractions in aqueous media of low ionic strength in contrast to methods of isolation of A-substance which rely on separations by means of organic solvents (19, 24) or high salt concentrations (24). The principal observation previously concerned with the direct separation of components in aqueous solution was in connection with the electrodialysis of mucin suspensions (4, 15); in that work, however, the solid separating after electrodialysis contained considerable

impurity and possessed low A activity compared to other fractions. A related observation was made by Morgan and King (24) who found that after heating a dilute acetic acid solution of A-substance and subsequent fractionation with ammonium sulfate (70-85% saturated) a water-soluble and a water-insoluble material were isolated on dialysis. The water-insoluble material was about as active as the original material in the hemolysis inhibition test and the water-soluble material was degraded judging from the results in the isoagglutination inhibition test.

Among the observations which were of importance in leading to the development of the new fractionation procedure were the following: 1) the extinction curves of preparations were sensitive to pH, values usually increasing on lowering the pH (Part III), 2) the effectiveness of electrodialysis in separating insoluble components was sensitive to the initial salt concentration and pH of solutions (Part III), and 3) on acidifying A-substance in aqueous solution (Part IV) an opalescence appeared which disappeared on titrating with base to pH 10 and which did not reappear on reacidifying the solution. The evidence suggested an aggregation of material in solution exhibited by turbidity or opalescence, which was strongly dependent on pH and ionic strength. Accordingly, a systematic study of these two variables was made with the aid of spectrophotometric analysis to determine the optimum conditions for appearance of turbidity and ultimately for the fractionation of the potent materials obtained by alcohol precipitation and electrodialysis (Part IV).

Experimental

1. Analytical and Serological Methods

Most of the methods used were described previously (Part IV, Experimental). The equivalent percent galactose was determined by a modification (see Part IX) of the procedure of Gurin and Hood (33). The colors obtained with carbazole were calculated in terms of galactose, the principal hexose present in A-substance (20, 25). In as much as the carbazole reaction occurs for other hexoses and pentoses, and because various factors influence the intensity of colors (33), the significance of the results is mainly qualitative. The results reported are the means of duplicate determinations; deviations from the means amounted to about ± 2% equivalent galactose.

2. Isolation Procedures

The most active fractions obtained by alcohol precipitation and electrodialysis (Part IV), fractions R18-F10 and R18-F11, were used as starting materials.

Separation of components in fraction R18-F10. To 10 ml of A-substance solution, 5.08 mg/ml, was added 1 ml of 0.012 M HCl. The resulting solution had a pH of 3.2 and was 0.00109 M HCl, and contained 4.62 mg A-substance per ml. On acidification the solution became quite turbid and on standing in the cold room overnight a gelatinous precipitate settled out which amounted to about 0.3 ml after centrifuging. The supernatant was decanted and a portion lyophilized; the precipitate was washed once with 10 ml of 0.00109 M HCl, and a portion lyophilized. The precipitate accounted for about 15% of the original solid in solution and the supernatant for the remainder. The

precipitate suspended in 11 ml of 0.00109 M HCl could be dissolved by the addition of one drop of 0.9% saline or one drop of 0.15 M $BaCl_2$. The precipitate disperses and goes into solution on addition of water.

Separation of components in fraction R18-F11 (Run 19). To one liter of aqueous solution containing 5.59 g of R18-F11 was added 100 ml of 0.012 M HCl. The solution, pH 3.1 and 0.00109 M HCl, became quite turbid and after centrifuging at 1500 RPM for about one hour a well-packed precipitate and a slightly turbid supernatant was obtained. The supernatant was decanted and lyophilized; this fraction, R19-F1, amounted to 4.25 g (7%). Since this fraction gave slightly acid solutions after lyophilizing, an aqueous solution was dialyzed several days, and the dialyzed material after lyophilizing assigned the number R19-F1A.

The precipitate which amounted to 60 ml was washed four times with 110 ml of 0.00109 M HCl by suspending in solution and centrifuging. After three washings the precipitate settled rapidly in a few minutes as gelatinous particles which adhered to one another on standing. The particles under the microscope appeared as globular masses which possessed no evident crystalline character. The washings were each lyophilized separately and amounted successively to 150, 52, 34 and 28 milligrams of solid. The residue after the four washings, about 30 ml of precipitate and solution, was quite turbid and viscous. After addition of only 50 ml of water the solution appeared homogeneous, viscous, and noticeably less turbid; no precipitate separated from this solution. The solution was lyophilized and 0.83 g (15%) of solid

was obtained (fraction R19-F2). A dialyzed portion of R19-F2 was assigned the number R19-F2A. The total recovery of solid material in this procedure was 96%.

Separation of components in fraction R18-F11 (Run 20). This procedure was similar to that for Run 19 except that the insoluble component was reprecipitated rather than washed. The insoluble material separating on adjusting a solution of 21.3 g of R18-F11 to pH 3 (0.00112 M HCl, 3.3 liters) was centrifuged off. It was found by preliminary experiments that the insoluble component could not be centrifuged readily from solutions which contained A-substance at concentrations of 1% or 2%. The slightly turbid supernatant was decanted, dialyzed five days in the cold room, and then lyophilized. solid amounting to 14.1 g (66% was isolated (fraction R20-F1).

The precipitate, about 225 ml, was reprecipitated five times by dissolving the precipitate each time in 500 ml of water, adding 50 ml of 0.012 M HCl, and then centrifuging. The solutions were 0.0011 M HCl and had pH of 3.0-3.1.

This procedure effectively yielded a precipitate with a constant absorption spectra in the ultraviolet. Fig. 1 shows the absorption spectra of each of the supernatants obtained during reprecipitation; since the volumes of the total solution for the reprecipitation varied from 640-770 ml, the optical densities were recalculated to the common basis of one liter of solution. As may be seen, the spectra for the fourth and fifth supernatant are practically the same except below about 260 mu. As a result it is presumed that the precipitate also had reached a practically constant spectra at this point.

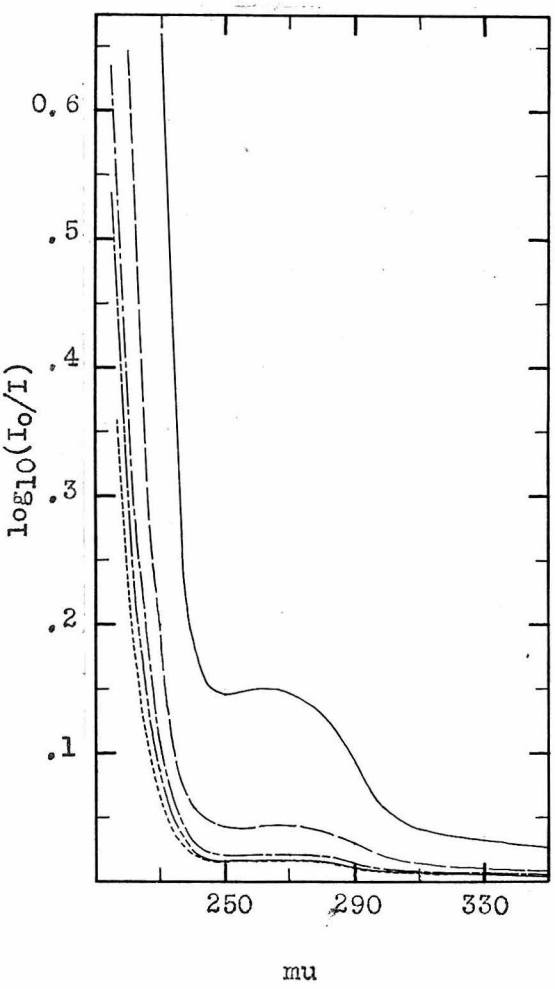


Fig. 1 . Optical densities of supernatants on reprecipitation of R20-F2 by adjustment of solutions to pH 3. ————, first supernatant; —— ——, second supernatant; ——·——, third supernatant; —— - - ——, fourth supernatant; - - - - - -, fifth supernatant. The curves are calculated to common basis of one liter of solution.

The supernatants from the first and second reprecipitation contained 0.86 g and 0.21 g of solid. The precipitate obtained after the fifth reprecipitation was dissolved in water, dialyzed four days (no chloride in dialysates), filtered to remove a few suspended particles, and finally lyophilized. Solid amounting to 3.00 g (14% was obtained (fraction R20-F2). A total recovery of 85% of the starting material was obtained in this procedure.

Results

1. Influence of pH and Ionic Strength on the Aggregation and Separation of Insoluble Components in Aqueous Solution

The detection of acid-insoluble components and a suitable procedure for their separation in well-fractionated A-substance preparations was made possible through a quantitative spectrophotometric study of the effect of pH and ionic strength. Aqueous solutions of R18-F10 were prepared and to aliquots various hydrochloric acid-sodium chloride mixtures were added; the final A-substance concentration was 0.46%. In Fig. 2 the effect of adding increasing quantities of hydrochloric acid is shown. The solutions became turbid with decreasing pH until a maximum was attained in the region 3-3.5; thereafter on further decreases in pH the turbidity diminished and disappeared. As a consequence of the scattering and reflection of light due to the turbidity, the apparent extinction values of the solution showed corresponding changes. In Fig. 2 the extinction values at two wavelengths are given, 260 mu and 350 mu; at the shorter wavelength the solution possesses some specific absorption (see Fig. 3), while at the longer

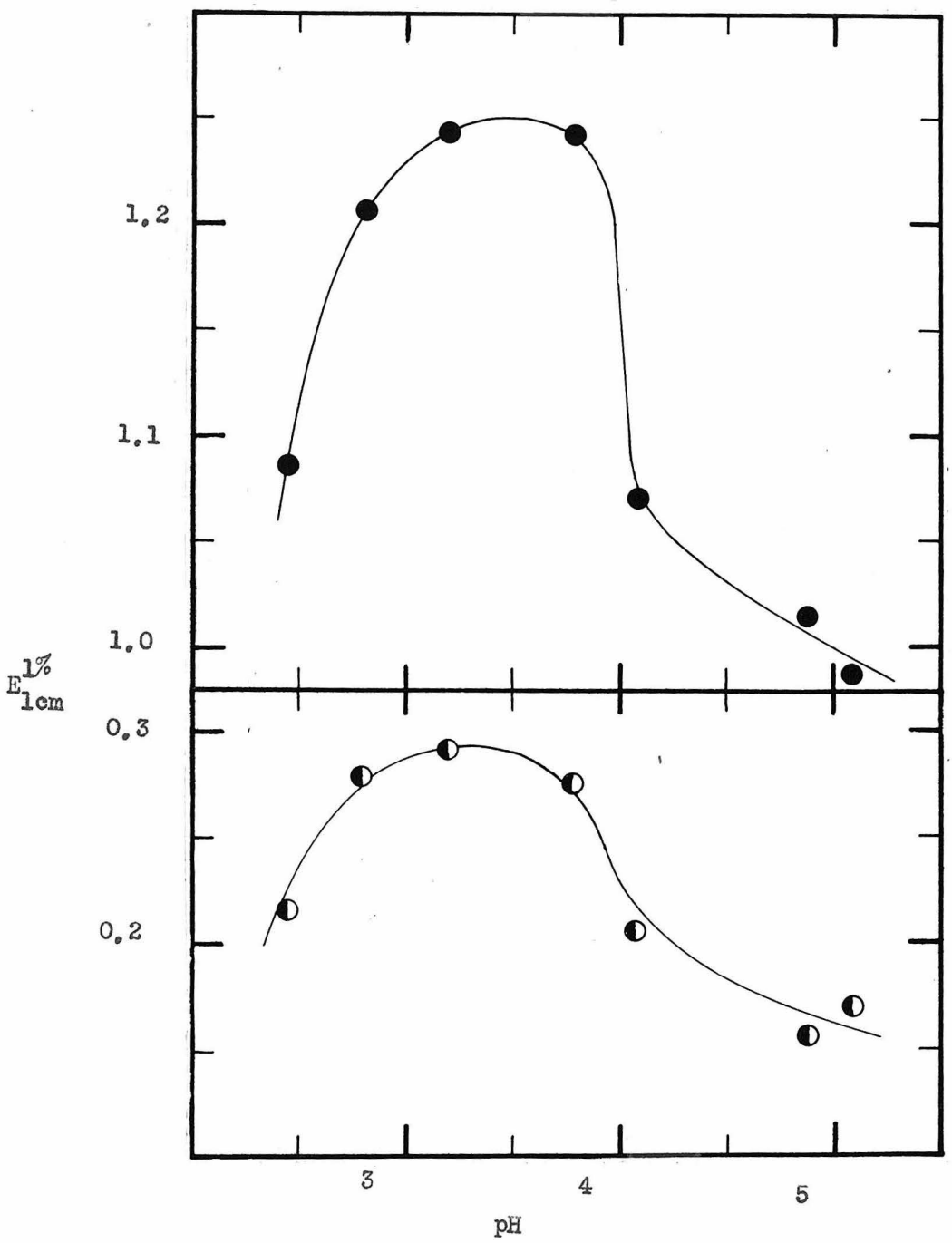


Fig. 2 . Effect of pH on extinction values for A-substance from hog gastric mucin (R18-F10): ● , 260 mu; ◐ , 350 mu. A-substance concentration, 4.62 mg/ml.

wavelength little or no specific absorption is apparent.

The added effect of ionic strength[1] is illustrated by the extinction curves in Fig. 3. The extinction curves are approximately the same at pH 5.5 when the ionic strength is varied from 0 to 0.0109. However at lower pH the effect of increasing the ionic strength is to depress the extinction curves. For example, at pH 2.8 the extinction values, $E^{1\%}_{1cm}$, at 260 mu, decrease successively, 1.21, 1.06, and 0.94 when the ionic strength is increased, respectively, 0.00218, 0.00436 and 0.0109. In fact at ionic strength 0.0109 the effect of decreasing pH on the extinction values is essentially absent. These results are in accordance with visual observations of the clarification of acidic solutions on the addition of neutral salts. The effect of ionic strength is no doubt of importance in decreasing the turbidity and thus the extinction values at pH less than 2.8 (Fig. 2).

The results above indicate the complex way in which the aggregation of material in fraction R18-F10 is affected by pH and ionic strength. It is apparent further from the spectra shown in Fig. 4 that the ease of separation of aggregated components is similarly affected by these two variables. The spectra of the solutions described in Fig. 3 were measured immediately after the adjustment of pH and ionic strength. In Fig. 4 the optical densities, $\log_{10}(I_o/I)$, of these solutions are compared with the densities obtained after the solutions had been standing in the cold room (5°) overnight and then,

---

[1] The ionic strength was calculated from the added hydrochloric acid and/or sodium chloride. The contribution of A-substance to the ionic strength, due to the small amount of ash present, is not included.

Fig. 3 . Effect of pH and ionic strength on extinction curves of A-substance from hog gastric mucin (Fraction R18-F10, 4.62 mg/ml).

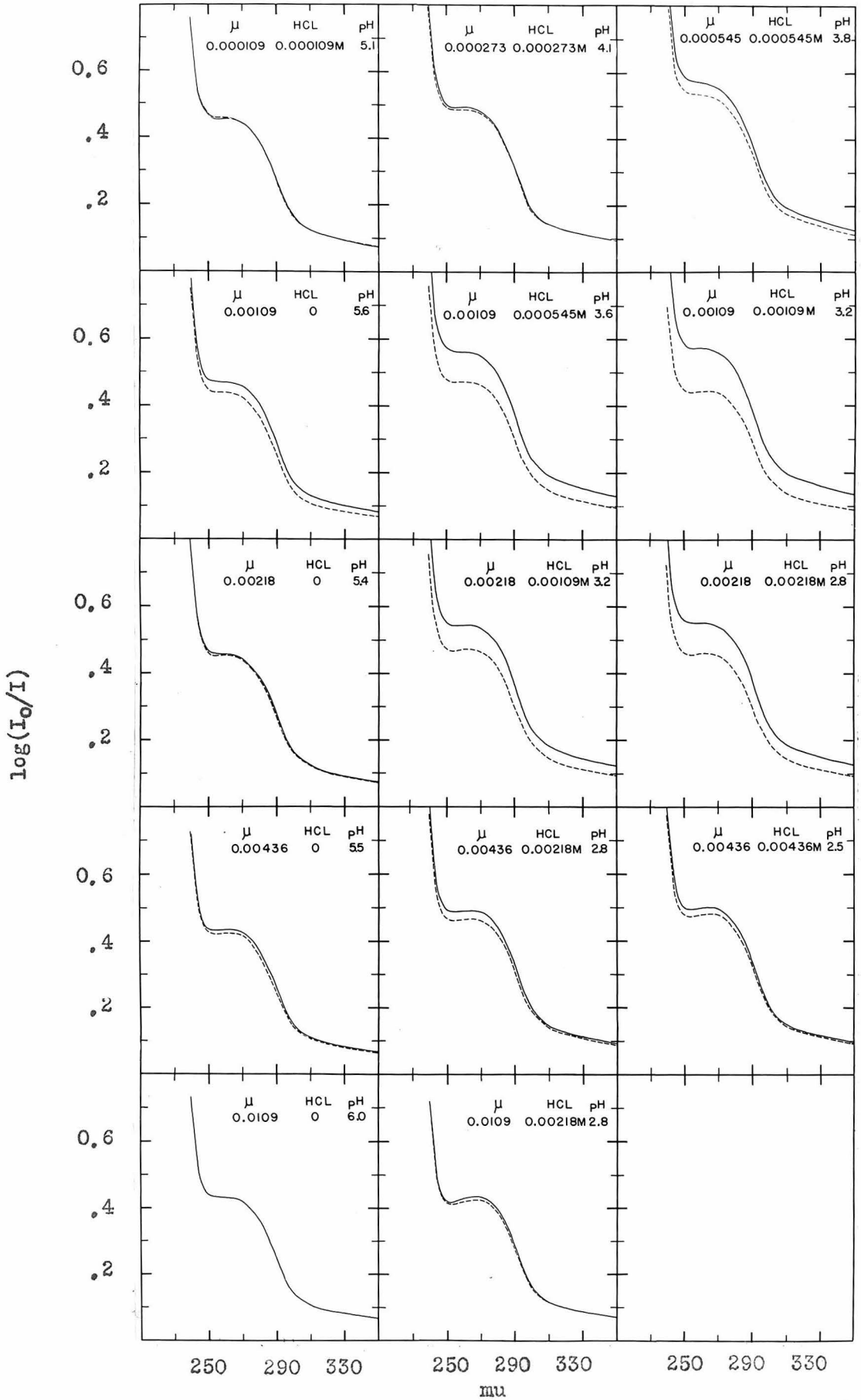


Fig. 4 . Absorption spectra of A-substance from hog gastric mucin (R18-F10, 4.62 mg/ml) before and after separation of insoluble component: ———, spectra of solutions immediately after adjustment of pH and ionic strength; ------, spectra of supernatants after standing and centrifugation.

in some instances, centrifuged. In those cases where substantial turbidities were apparent, separation of insoluble material occurred and the densities of the solutions showed appreciable decreases throughout the ultraviolet. It may be seen that maximal separation of insoluble material occurred for solution HCl 0.00109 - μ 0.00109 as judged from the decrease in optical densities. In the isolation procedure discussed above about 15% of the total material was insoluble under these conditions. The sensitivity of separations to pH is evident from solution HCl 0.000545 - μ 0.000545 and solution HCl 0.00218 - μ 0.00218 which were approximately as turbid as solution HCl 0.00109 - μ 0.00109 but the former solutions did not separate insoluble material as well. In those examples where no appreciable turbidities were evident, no precipitation occurred and the densities on standing showed very little change.

2. Immunological Properties of Soluble and Insoluble Components Obtained on Adjustment of A-substance Solutions to pH 3

Blood group A properties. The serological properties of the fractions - insoluble components R19-F2 and R20-F2, and soluble components R19-F1 and R20-F1 - obtained in Runs 19 and 20 were determined by the isoagglutination inhibition test (15) and the hemolysis inhibition test (4). The titers of the preparations were comparable even though results were often obtained on different days with different cells, since as has been the practice in this Laboratory the titers of control preparations were determined at the same time so that

results could be corrected to a common basis.

Results for the inhibition of isoagglutination of A cells by anti-A serum (B serum) are shown in Table I.

Table I

Isoagglutination Inhibition Titers of A-substance Preparations

Fraction	Titer[1] microliters serum neutralized per microgram
R19-F1	130
R20-F1	150
R19-F2	160
R20-F2	185

The acid-insoluble components, R19-F2 and R20-F2, have the same A activity as the soluble components, R19-F1 and R20-F1, within the limits of experimental error (approximately ± 40 microliters of serum neutralized per microgram for these results). The fractions have 5-6 times the potency of Wilson mucin (Part IV).

Results for the inhibition of hemolysis of sheep cells by anti-human A cell rabbit immune sera are shown in Fig. 5 and the hemolysis inhibition titers are summarized in Table II. The results are presented in detail since this test indicates that the insoluble

[1] Mean of duplicate determinations

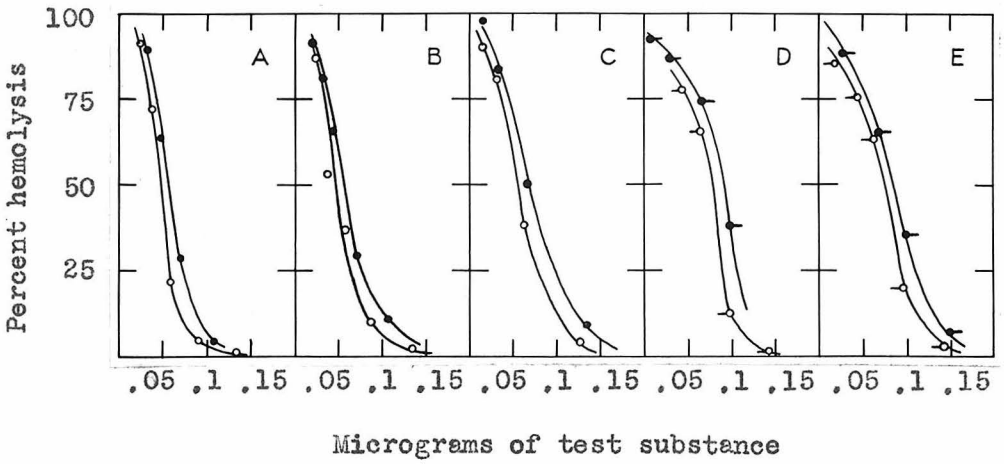


Fig. 5 . Hemolysis inhibition curves for A-substance preparations. Acid-soluble components, ●— R19-F1 and ● R20-F1; acid-insoluble components, —○ R19-F2 and ○ R20-F2.

Table II

Hemolysis Inhibition Titers of A-substance Preparations

Run[1]	Titer, micrograms inhibiting 50% hemolysis							
	Acid-soluble components				Acid-insoluble components			
	R19-F1		R20-F1		R19-F2		R20-F2	
	obs.	corr.	obs.	corr.	obs.	corr.	obs.	corr.
A			0.057	0.059			0.049	0.051
B			.058	.060			.050	.052
C			.067	.060			.056	.050
D	0.090	0.065			0.074	0.054		
E	.084	.061			.075	.055		

1 Data from above figure.

component has significantly greater potency than the soluble component. The soluble components, R19-F1 and R20-F1, have titers of 0.063 and 0.060 micrograms, respectively, while the insoluble components, R19-F2 and R20-F2, have titers of 0.055 and 0.051 micrograms respectively. The reproducibility of this difference and the precision of the hemolysis test indicate that the difference is significant. The soluble and insoluble components have, respectively, about 4.5 and 5.5 times the potency of Wilson mucin in this test.

Blood group O properties. Results for the inhibition of agglutination of blood group O cells by selected cattle sera are shown in Table III; additional isoagglutination inhibition titers towards A

Table III

Serological Specificity of A-substance Preparations

Fraction	Agglutination inhibition titers Anti-O x 10^3	Anti-A x 10^3
C-135	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

cells are also given[1]. The fractions R19-F1A and R19-F2A were isolated

[1] We are indebted to Dr. W.T.J. Morgan for the results in Table III.

from R18-F11, while C-135 corresponds to the centrifuged mucin suspension used to isolate R18-F11 (Part IV). The separation of A and O components is evident since the acid-soluble and acid-insoluble components have two to four times the A activity of C-135 while having only one-fourth to one-half the O activity. While the acid-insoluble component would appear to be purer than the acid-soluble component with respect to A-substance - the former contains less O-activity and greater A activity - the precision of the agglutination test is so low that the difference is probably not significant. R19-F1A and R19-F2A have only 6-12% of the O activity of O-substance isolated from human ovarian cyst fluid (see Table VII, Part IV).

3. Chemical and Physical Properties of Preparations

Chemical properties. Analytical data for the soluble (R20-F1) and insoluble (R20-F2) components obtained on adjusting a solution of R18-F11 to pH 3 are given in Table IV. The preparations appear quite similar except that the insoluble component possesses slightly more nitrogen. Results in the qualitative tests for hexose, arginine, and histidine are the same within experimental error; the equivalent N-acetyl glucosamine content of the insoluble component is greater than that of the soluble component. The insoluble component seems to possess slightly greater acid-combining capacity. Fractions R20-F1 and R20-F2 in 1% solution do not form gels on addition of an equal volume of 0.05 M borate buffer pH 8.5 (prepared by adding 6 M HCl to 0.05 M borate solution to pH 8.5). The formation of a gel under these conditions is presumably indicative of undegraded A-substance (24).

Table IV

Chemical and Physical Properties of A-substance Preparations

	Acid-soluble component R20-F1	Acid-insoluble component R20-F2
Total carbon, %	41.1	42.6
Total hydrogen, %	7.2	7.4
Total nitrogen (Dumas), %	5.1	5.6
Ash (residue on Dumas combustion), %	.8	.5
Equivalent arginine content, % (before hydrolysis)	.30	.29
Equivalent histidine content, % (before hydrolysis)	.48	.53
Equivalent N-acetylglucosamine, %	13.0	11.5
Equivalent galactose, %	31	26
Maximum tyrosine content, % (from spectral absorption)	1.0	.8
Maximum tryptophan content, % (from spectral absorption)	.3	.2
Base-combining capacity from pH 3 to 8, moles x 10^5 per gram preparation	11	10
Acid-combining capacity from pH 8 to 10.5, moles x 10^5 per gram preparation	4	7

Table IV.-(concluded)

		Acid-soluble component R20-F1	Acid-insoluble component R20-F2
Optical rotation (c 1%, phosphate buffer pH 8.0, μ 0.136), α_D^{25}		+ 1 ± 2	+ 19 ± 2
Extinction coefficients (citrate-phosphate buffer pH 7.3), $E_{1cm}^{1\%}$	at 260 mu	1.17	0.87
	at 350 mu	.13	.14
Viscosity increment[1], ν_o		230	275
Electrophoretic mobility (acetate buffer pH 3.85, μ 0.132, 1.35°C), $cm^2 volt^{-1} sec^{-1} \times 10^5$, anodic		1.1	1.1 (main component)
			3.0 (fast component)
Molecular size			
Light scattering[2]		0.9×10^6	1.1×10^6

[1] ν_o obtained by extrapolation of viscosity increment, $\nu = (t/t_o - 1)1/\Phi$, to zero concentration, ν_o, and then extrapolating ν_o to zero average shear gradient.

[2] Data on the similar fractions R19-F1A and R19-F2A corresponding, respectively, to fractions R20-F1 and R20-F2.

Optical rotation. The insoluble component, R20-F2, exhibits considerably larger positive rotation, + 19°, than the soluble component, + 1° for R20-F1, (Table IV). The magnitude of these results is reasonable in view of their relative yields from R18-F11 and the low rotation of +3° of R18-F11. The relative rotations are in agreement with results at a different wavelength and unspecified solutions obtained by Dr. W.T.J. Morgan on the similar preparations R19-F1A (acid-soluble), R19-F2A (acid-insoluble), and R18-F11 (parent compound), $[\alpha]_{5461}$, respectively, +10°, +32°, and +14°.

Ultraviolet absorption spectra. The insoluble components at pH 3, fractions R19-F2 and R20-F2, show less extinction in the ultraviolet than the corresponding soluble components, R19-F1 and R20-F1 (Fig. 6). The insoluble components obtained by either washing of precipitated material, R19-F2, or by successive reprecipitations, R20-F2, have essentially the same extinction curves. Both fractions show a small amount of specific absorption in the region 250-280 mu. The extinction curves of the soluble components, R19-F1 and R20-F1, differ somewhat more markedly.

The spectral absorption at 280 mu has been used (for method, see Part III) to calculate the maximum tyrosine and tryptophan content of preparations (Table IV).

Electrophoresis. Electrophoresis diagrams of preparations R20-F1 and R20-F2 are given in Fig. 7 and the mobilities of boundaries at pH 3.9 are summarized in Table IV. R20-F1, the soluble component at pH 3, has a low anodic mobility and appears to be homogeneous. R20-F2, the insoluble fraction, however is heterogeneous. The

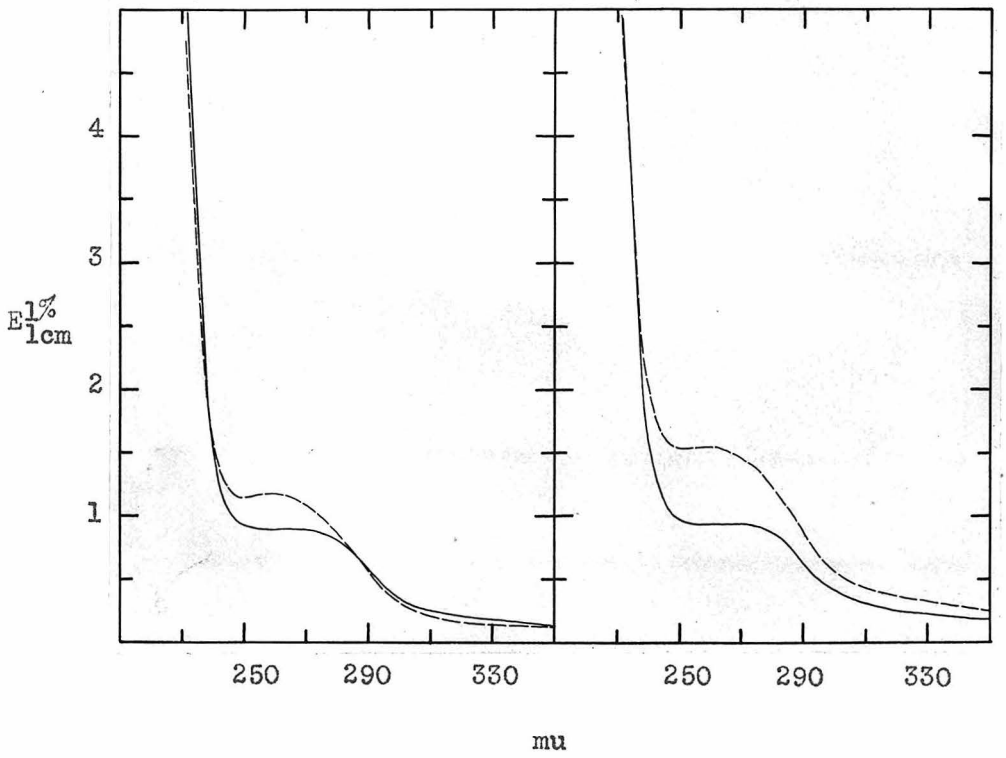


Fig. 6 . Extinction curves of soluble and insoluble components of A-substance obtained on adjustment of aqueous solutions to pH 3 (0.00109 M hydrochloric acid). Left: ——— ———, R20-F1; ————, R20-F2. Right: — —, R19-F1; ————, R19-F2.

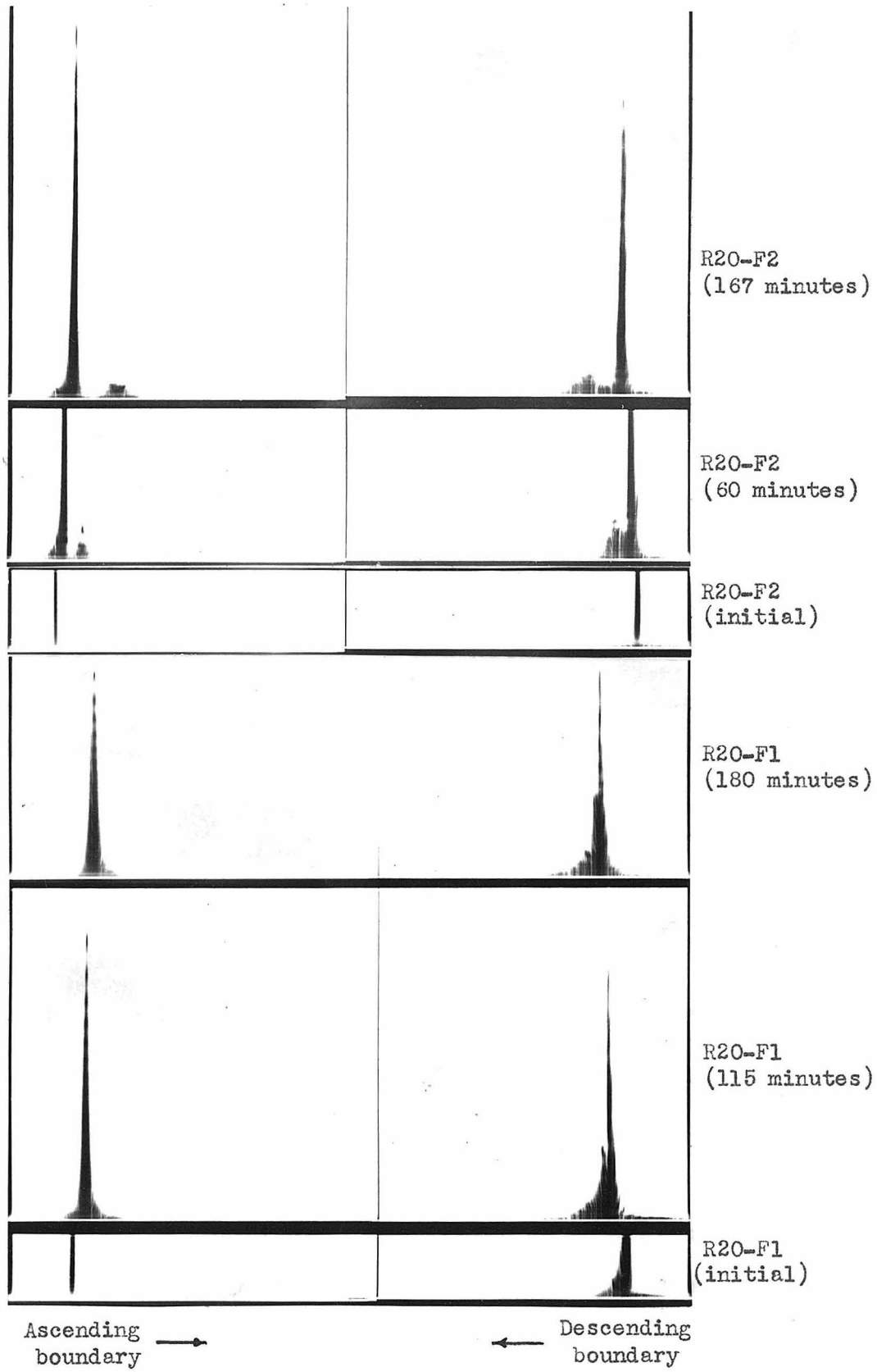


Fig. 7 . Electrophoresis diagrams of R20-F1 and R20-F2. (0.5% solutions, 1.35°C, acetate buffer pH 3.85, μ 0.132).

fraction comprises at least two components having anodic mobilities; the fast component amounts to 10-15% of the total material on the basis of relative areas. The main component in R20-F2 and the single component in fraction R20-F1 have essentially the same mobility as fraction R18-F11, the parent compound. However, as may be seen from Fig. 14, Part IV, there is no evidence for a fast component in fraction R18-F11. The failure to detect the fast component may be due to its low content in R18-F11, calculated to be about 2%, or possibly due to some interaction of components. It should be pointed out that the detection of the fast component in R20-F2 is one of the few instances in which electrophoresis has been successful in indicating heterogeneity in A-substance preparations; results obtained by Mr. David H. Brown and Mr. Edward L. Bennett in this Laboratory indicate that even only moderately potent A-substance preparations often appear essentially homogeneous by electrophoresis (for example, see fraction C-127, Fig. 14, Part IV).

Viscosity and molecular size. These data (Table IV) have been obtained by Mr. Arthur Pardee and Mr. Robert Blaker (27). The acid-soluble and acid-insoluble components have similar viscosity increments and molecular weights by light scattering (weight average molecular weight 0.9 - 1.1 x 10^6). The number average molecular weight of A-substance (fraction R18-F10, see Part IV) appears to be about one-fourth the weight average. The viscosity increment of A-substance decreases on increasing the average shear gradient and the significance of this observation has been discussed by Mr. Pardee (27) in suggesting a rod-like shape for A-substance (axial ratio 60/1).

Electron micrographs of fraction R20-F2 by technique of gold-shadowing on mica sheets have been made by Mr. Burton Henke of the Physics Department of the Institute. Preliminary results indicate large aggregates of material as well as small particles, ca 200 Angstroms diameter, indicating a maximum molecular size of about 3×10^6. This estimate is uncorrected for contribution to size by gold accumulated by particles. In certain micrographs rod-like structures are suggested although the difficulty of resolving small particles from the gold film background requires further work for the verification of this feature.

Solubility of acid-insoluble component at pH 3 and low ionic strength. The approximate solubility of R20-F2 was determined by adjusting aqueous solutions at room temperature, about 25°, containing varying amounts of the preparation to 0.00109 M HCl (solutions at pH 3.01 $\pm$ 0.02, except the most concentrated A-substance solution which had a pH of 3.08) and then centrifuging the resulting mixture for 15-20 minutes in a clinical centrifuge. Visual observations as to the occurrence of turbidity on acidification and resulting clarification on centrifuging were made and then the ultraviolet absorption spectra of the supernatants were determined.

In Fig. 8 the extinction values at various wavelengths are plotted as a function of the total concentration of solid material added. The resulting curves show typical breaks at the occurrence of solid phase (about 0.1 mg/ml); however, the extinction values increase with greater quantities of solid phase (42) and thus indicate that R20-F2 is heterogeneous as to solubility at pH 3. It is apparent that

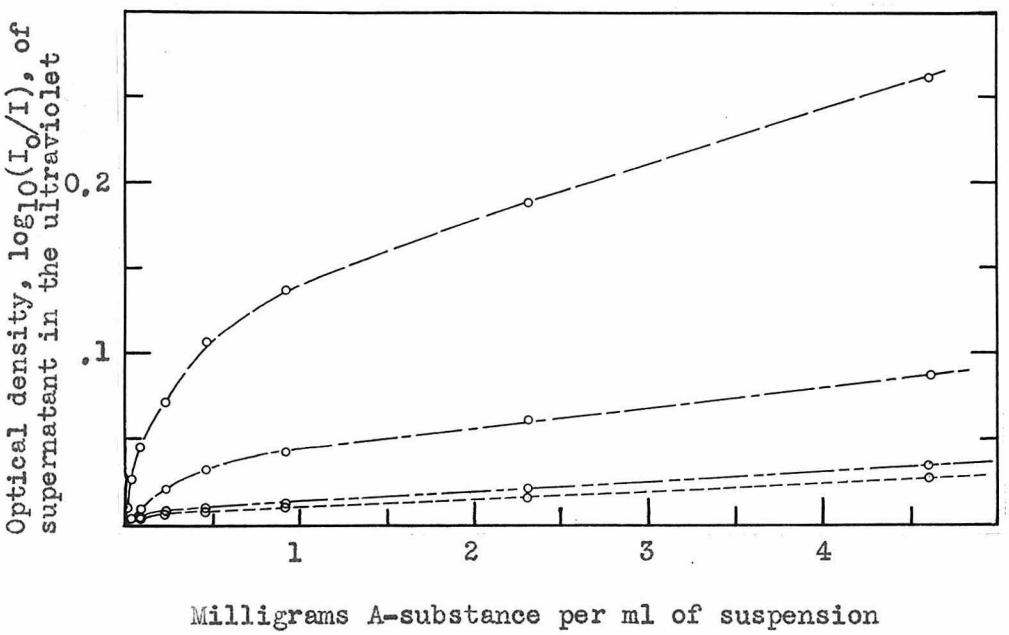


Fig. 8 . Solubility curves of A-substance (R20-F2) in solutions at pH 3 (0.00109 M hydrochloric acid). ——— ———, densities at 220 mu ; ——— - ———, densities at 230 mu ; ——— - - ———, densities at 240 mu ; --------, densities at 260 mu .

heterogeneity only as to components having absorption in the ultraviolet is detectable by this experiment.

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

4. General Applicability of Fractionation of A-substance Preparations by Adjustment of pH and Ionic Strength

The possibility of separating purified A-substance into two fractions differing as to solubility at pH 3 led to a qualitative examination of the applicability of this procedure to a number of other A-substance preparations from mucin and pepsin, and A active and inactive preparations from individual hog stomach linings. Aqueous solutions of fractions were prepared and 0.012 M HCl or 0.012 M NaCl was added. The final solutions were 0.00109 M in salt or acid; the pH of the salt solution varied from 5.5 to 7, while, due to the presence of buffering materials, in some preparations the pH of the acid solutions varied from about 3 to 4. The amount of turbidity occurring on acidification was conveniently measured by the ratio, t, of the extinction value at 350 mu of solutions containing acid to the value for the same solution in which salt replaced acid. A value of t greater than about 1.1 indicates that a significant turbidity occurred on acidification. Similarly the separation of insoluble material was measured by the ratio of the extinction value at 350 mu immediately after adding $\frac{\text{salt}}{\text{or acid}}$ to the value obtained after the solution was centrifuged. These ratios are indicated by s_{NaCl} and s_{HCl}

where the subscript designates the particular solution concerned. Values of $\underline{s}$ greater than 1.1 indicate that significant amounts of material were separable after adding acid or salt. Results which have been obtained for a number of preparations are given in Table V.

The marked difference between the soluble component, R20-F1, and the insoluble component, R20-F2, occurring in fraction R18-F11 is indicated by the value of $\underline{s}_{HCl}$ of 1.3 and >25 respectively. The insoluble component had not been completely removed from R20-F1 since a small precipitate was still observed on acidification. A number of fractions obtained in the course of preparing fraction R18-F11 were examined for insoluble components. R18-F2 (see Part IV) which is one of the early main components leading to fraction R18-F11 contains appreciable insoluble component, ($\underline{s}_{HCl}$ 2.0); the fractionation by pH adjustment is then apparently applicable quite early in the fractionation of mucin. However, the presence of contaminants may influence the separation of insoluble material since fractions R18-F6A, R18-F9C, R18-F12, and R18-F13 which were obtained as less active fractions in the preparation of R18-F11, exhibit turbidity on acidification, $\underline{t}$ 1.2-2.6, but only fractions R18-F6A and R18-F9C show appreciable separation of insoluble material, $\underline{s}_{HCl}$ 1.2-1.3.

The occurrence and separation of insoluble material in A-substance preparations is not apparent in case formamide treatment (fraction R7-F4A) or the procedure of Meyer, Smyth, and Palmer (25) involving preliminary alkaline degradation (fraction C-47) have been applied. These procedures are known to produce degraded A-substance (4, 19, 24). It is of interest that the A-substance degraded by papain - HCN

Table V

Effect of Addition of Acid on the Aggregation and Separation of Insoluble Components from A-substance Preparations

Source	Procedure	Reference (Part)	Fraction	Conc. mg/ml	pH	$\underline{t}$	s_{NaCl}	s_{HCl}
Hog mucin	Alcohol fractionation and electrodialysis	V	R20-F1	1.82	3.0	1.7	1.0	1.3
			R20-F2	.54	3.0	2.8	1.0	>25
		IV	R18-F2	1.82	3.2	1.4	1.1	2.0
			R18-F6A	4.41	3.5	2.6	1.1	1.2
			R18-F9C	4.75	3.1	1.4	1.1	1.3
			R18-F12	4.38	4.5	1.2	1.0	1.0
			R18-F13	4.83	3.3	1.4	1.1	1.0
	Formamide treatment	I	R7-F4A	1.82	3.0	1.0	1.1	1.2
	Papain-HCN treatment	II	R6-F5A	1.82	3.1	1.6	1.1	1.7
	Alkaline digestion	II	C-47	1.82	3.2	.9	1.1	1.1
Pepsin	Alcohol fractionation	II	C-22	1.82	3.2	1.4	1.0	1.9
	Autolysis and alcohol fractionation	II	C-26	1.82	3.3	.9	1.1	1.0

Table V.-(concluded)

Source	Procedure	Reference (Part)	Fraction	Conc. mg/ml	pH	$\underline{t}$	s_{NaCl}	s_{HCl}
Hog stomach lining	Peptic digestion and alcohol precipitation	VI	H1-F3*	1.82	4.2	0.7	1.1	1.1
			H2-F1	1.82	4.4	.9	1.0	1.0
			H3-F1	1.82	3.9	.9	1.2	1.0
			H4-F1*	1.82	4.0	1.1	1.1	1.2
			H5-F1	1.82	3.8	.9	1.1	1.1
			H6-F1	1.82	3.8	1.8	1.1	.9
			H5A-F1	1.73	5.0	4.0	1.1	1.0
Hog mucin + hog stomach lining	Mixing experiments		H1-F3* + R20-F2	1.27 + .54	3.8	.8	1.2	1.0
			H2-F1 + R20-F2	1.27 + .54		.9	1.0	
			H3-F1 + R20-F2	1.27 + .54	3.6	1.6	1.2	1.1
			H4-F1* + R20-F2	1.27 + .54	3.7	2.5	1.1	1.0

* Preparations isolated from individual linings not possessing A-specific factor.

(4, 19), on the other hand, still exhibits acid-insoluble material (fraction R6-F5A, s_{HCl} 1.7).

The A-substance fractions obtained from Wilson pepsin by alcohol precipitation (C-22) or by autolysis followed by alcohol precipitation (C-26) differ substantially in the separation of insoluble material on acidification. Fraction C-22 exhibits turbidity, $\underline{t}$ 1.4, and precipitation of insoluble material, s_{HCl} 1.9, while C-26 shows none of these properties, $\underline{t}$ 0.9 and s_{HCl} 1.0. While the difference between C-22 and C-26 may be due to the action of pepsin on the insoluble component it has been found that peptic digestion of the acid-insoluble fraction R20-F2 from mucin did not alter the acid-insoluble property. Thirty-two mg of R20-F2 in 10.5 ml of 0.1 M citric acid (pH 2.1) was digested for three days at 37° in the presence of 0.5 mg of Plaut pepsin. The solution on dialysis gave a precipitate in a short while which partially dissolved on longer dialysis. The supernatant contained insoluble material on acidification with hydrochloric acid. A control containing R20-F2 and no pepsin behaved similarly.

Unlike A-substance from hog mucin, preparations isolated from individual hog stomach linings by peptic digestion followed by alcohol precipitation generally do not become turbid on acidification, e.g., the A active preparations, H2-F1, H3-F1, and H5-F1, and the A inactive preparations H1-F3 and H4-F1 have values of $\underline{t}$ of less than 1.1. Some of these preparations have values of $\underline{t}$ which indicate that clarification has occurred (H1-F3). The A active preparation H6-F1 differs considerably from the others in forming a turbid solution on acidification ($\underline{t}$ 1.8). It is of interest that H5A-F1 which was obtained

from the same lining as H5-F1 by acid extraction differs remarkably from the latter on addition of acid, - a quite intense turbidity is evident, t 4.0, which increases on further acidification (H5A-F1 as well as other fractions from linings contains considerable buffering material). None of the above preparations, however, precipitates appreciable insoluble material from acid solution; most of the preparations give very small amounts of precipitate in acid as well as salt solution.

In order to test whether the lack of acid-insoluble material in the hog lining preparations was due to absence of this component or to other factors which interfere with its detection several mixing experiments were tried. Fraction R20-F2 the acid-insoluble component from R18-F11 was mixed in aqueous solution in turn with fractions H2-F1, H1-F3, H3-F1 and H4-F1, and then acidification performed. In two instances, H1-F3 and H2-F1, no increase in turbidity, t 0.8 and 0.9, was noticed over that in the absence of R20-F2, t 0.7 and 0.9; H3-F1 and H4-F1, however, showed marked increases of turbidity, t 1.6 and 2.5, over that in the absence of R20-F2, t 0.9 and 1.1. No insoluble component separated, however, from these solutions. It is obvious from these results that considerable interaction of acid-insoluble components with other material is possible in some preparations so that the absence of turbidity on acidification cannot be taken to be due to the absence of acid-insoluble components. It is significant that the occurrence of a turbidity on mixing R20-F2 with the A active material H3-F1 and the A inactive material H4-F1 indicates that R20-F2 isolated from hog mucin is substantially different from the material

which occur in the preparations from hog linings.

Discussion

The detection and isolation of soluble and insoluble components in purified A-substance preparations from hog gastric mucin by adjustment of aqueous solutions to pH 3 and at minimum ionic strength is described. The insoluble component is obtained in about 15% yield from A-substance preparations purified by electrodialysis and alcohol precipitation; in terms of hog mucin the yield of insoluble component is about 0.9%. This component is soluble in water but its solubility at pH 3 (0.00109 M HCl) in the absence of added salt is less than 0.1 gram per liter; the insoluble component separates in acid solution as gelatinous particles with no obvious crystalline character. Like many proteins whose solubilities increase on increasing the ionic strength (42) the insoluble component dissolves on addition of salt; at pH 2.8 and ionic strength of 0.0109 the solubility is greater than one gram per liter (Fig. 4). The unique solubility properties of this component are not altered by the action of crystalline pepsin.

The soluble and insoluble components possess equivalent capacity within experimental error for inhibiting the isoagglutination of blood group A cells (15). The insoluble component, however, appears to be about 15% more active than the soluble component in the inhibition of hemolysis of sheep cells by anti-human A cell rabbit immune sera (4). Since the activites of the components in the isoagglutination test are commensurate with those obtained in the hemolysis test (about five times the potency of Wilson mucin) it is concluded that the

preparations are not degraded on this criterion; the activity in the former test is known to decrease under various conditions of degradation (4, 19, 24). However, neither component forms a gel with borate buffer pH 8.5 in the test of Morgan and King (24) which presumably would indicate the preparations are degraded on this criterion. There is no evidence that the two components are related immunologically to the forms of A-substance found in human urine by Jorpes and Thaning (56), one of which possesses capacity to inhibit isoagglutination and the other the capacity to inhibit lysis of sheep cells.

The results in Table IV also indicate that the two components resemble each other chemically and physically. Analytical data and color tests for arginine, histidine, hexose, and N-acetylglucosamine, viscosity, molecular size, and base-combining and acid-combining capacities are similar. The more potent acid-insoluble fraction has a more positive optical rotation and smaller absorption in the ultraviolet than the acid-soluble fraction. It is not possible at present to discuss the ways in which some of the small differences in the properties of the two forms could account for their striking difference in solubility at pH 3 in as much as both forms are known to be heterogeneous by various criteria. The insoluble from is heterogeneous by electrophoresis (15% of fast-moving component, Fig. 7) and solubility (Fig. 8). Both forms are heterogeneous on the basis of serological specificity, being contaminated with small quantities of O-substance (see Part IV for discussion concerning O-substance in mucin); the insoluble component, for example, has been found to have about four times the A activity and one-fourth the O activity of the water-soluble solids of mucin.

Heterogeneity is also suggested by molecular size studies since weight and number average molecular weights disagree. Furthermore the acid-soluble component is known to be contaminated with a small amount of the acid-insoluble component.

It has generally been assumed that a single species of polysaccharide-amino acid complex has constituted A-substance in hog stomachs. This assumption was supported by results which showed preparations to be homogeneous by electrophoresis (24) or by the ultracentrifuge (19). These criteria would appear to be misleading since the results presented above have shown that the acid-insoluble component is separable in 15% yield from a preparation homogeneous by electrophoresis. While the two forms of A-substance which have been isolated appear similar in many respects it is conceivable that the small differences which do exist might be accentuated or other differences might appear when more homogeneous preparations of each of the forms are isolated. Until such homogeneity is achieved it would appear difficult to interpret results on the constituent sugars and amino acids of preparations (2) in terms of the composition of A-substance. The occurrence of the two forms may also be of some importance in contributing to the difficulty in fractionating mucin (Parts II and IV) so that A activity is concentrated predominantly in a single fraction.

The existence of the two forms of A-substance has bearing on the significance of the precipitin test of Kabat and Bezer (34) for the determination of the absolute purity of A-substance preparations (3). In this test the purity of A-substance is assessed by determining the proportion of its glucosamine precipitated by anti-A sera. Since an

important solubility correction in the test is based on the assumption that only a single substance precipitates, it is conceivable that the two forms of A-substance in mucin could introduce uncertainty.

Preliminary experiments indicate that fractionation by pH adjustment may be readily applicable to hog gastric mucin. This procedure would also appear to be a useful characterization test since preparations from different sources, e.g. mucin, pepsin and hog stomach linings, and obtained by different methods often show quite distinct behavior in regard to the separation of insoluble material in acid solution. The occurrence of acid-insoluble components in preparations is conditioned by the procedures of isolation. For example, acid-insoluble components do not generally occur in hog linings which have been subjected to peptic digestion followed by alcohol precipitation. However, acid extraction of a lining followed by alcohol precipitation led to a preparation containing an acid-insoluble component (H5A-F1, Table V). It should be pointed out that the blood group A properties of insoluble components obtained in qualitative experiments (Table V) have not been verified. Preliminary results indicate that the lack of detection of acid-insoluble components in preparations (Table V) may be due not only to their absence but also to the interaction of the components with substances which prevent detection. It is apparent that the problem of the origin of the two forms of A-substance must await further work on methods of isolation of A-substance from individual hog stomach linings.

Summary

The detection and isolation of two forms of A-substance, one soluble at pH 3 and low ionic strength, and the other insoluble, is described. The fractions have about five times the potency of Wilson hog gastric mucin, the insoluble component appearing more potent. Both fractions are heterogeneous by various criteria, including solubility, serological specificity, molecular size, and electrophoresis. A number of chemical and physical properties of the preparations are reported.

The significance of the two forms of A-substance in respect to the characterization and isolation of A-substance from hog stomachs is discussed.

VI Observations on the Isolation and Properties of Blood Group A Substance from Individual Hog Stomach Linings

The investigations of Witebsky (29), Bendich, Kabat, and Bezer (3), and Aminoff, Morgan and Watkins (1) have led to the finding that hog stomachs yield similar high molecular weight complexes having either blood group A or O properties. As a result isolation and chemical studies on preparations isolated from pooled hog stomach sources, e.g., mucin or pepsin, are complicated by the admixture of two similar but distinct components. The striking similarity of the A and O substances with regard to elementary composition, viscosity and reducing sugar and glucosamine content (3) does not, however, extend so far that relatively simple procedures (ref. (1), and Parts IV and V, this thesis) are unable to effect a partial separation. Despite the fact that the A and O components are separable in mucin, which suggests an as yet unrevealed chemical or physical dissimilarity in these substances, it now seems clear that progress on the characterization of the materials occurring in hog stomachs would be expedited by beginning with individual linings. Mucin, because of its ready availability, would still appear to be useful for studying differences in A and O substances and for the accumulation of large quantities of the active products. In this section preliminary experiments are reported on the isolation of substances from individual hog stomach linings by peptic digestion (3). One aim of this work has been to eliminate the possiblity that the extensively fractionated A-substance from hog mucin (see Parts IV and V) is contaminated with large amounts of O-substance; this would be revealed by a comparison of the potency of preparations isolated from individual linings and from mucin. During the course of the isolation it was possible to extend the

observations previously made (1, 3) on the A-substance from individual linings and also to devise a simple procedure for its detection.

Experimental

Preparation of A-substance from individual hog stomach linings. Hog stomachs, obtained from Cudahy Co., Los Angeles, were washed freely with water and then packed individually on ice overnight. The stomach linings were stripped off with the aid of a scalpel and then ground individually with 100 ml of water in a Waring blender. About eight volumes of 95% ethanol were added to the minced lining and after standing overnight in the cold room, the suspensions were filtered, and the solid lyophilized. The dried preparations were ground in a meat grinder and then in a Wiley mill to pass a No. 40 screen. The yields of dried linings are shown in Table I.

The peptic digestion of the linings was carried out essentially as recommended by Bendich, Kabat, and Bezer (3). The linings (10 g) were suspended in 0.1 M citric acid (75 ml) under toluene plus sufficient concentrated hydrochloric acid to bring the pH to 2.2 - 2.3. One milligram of crystalline pepsin (Plaut Laboratories, Lehn and Fink Co., Bloomfield, N.J.) was added and the solutions incubated at 37°. The pepsin showed very slight A activity in the isoagglutination inhibition test (15); it is estimated that less than 0.01% of A-substance was present. The major part of the hydrolysis occurred overnight as judged by nuetralization of acid and by the marked decrease in viscosity of the solution. The pH was adjusted back to 2.2 with hydrochloric acid after one day; only small increases in pH

Table I

Yields of Hog Stomach Linings

Hog No.	Weight of stomach lining (grams)
1	39.3
2	32.3
3	35.2
4	35.5
5	25.8
6	34.6
7	37.9
8	25.1
9	37.0
10	25.5
11	30.4
12	31.1

occurred thereafter. After three days another milligram of pepsin was added and the incubation continued for two more days. Then the solutions were centrifuged, the yellowish centrifugates and washings were reserved for the subsequent fractionation, and the residues dried at 110 - 120°. The residues amounted to 15 - 30% of the dried linings (Table II).

The centrifugates were fractionated first by precipitation with four volumes of ethanol; the supernatants were discarded. The

precipitates were taken up in water and any water-insoluble material centrifuged off and washed. The aqueous solution was then made 0.5% in sodium acetate and the precipitates separating on addition of two or three volumes of 95% ethanol reserved. The precipitates were suspended in water, dialyzed for several days in the cold room, a small amount of water-insoluble material centrifuged off and discarded, and the centrifugates lyophilized. The yields and the relative A activities of fractions in the isoagglutination inhibition test (15) and the hemolysis inhibition test (4) are summarized in Table II. The activities are calculated assuming Wilson hog gastric mucin (Item no. 443) possesses unit activity. All the relatively inactive preparations showed a small amount of inhibition in both tests at the highest concentration tested.

Detection of A-substance in hog stomach linings. About 10 mg of the stomach lining was added to 0.5 ml saline and 0.5 ml of a diluted solution of the appropriate serum. The anti-A and anti-B sera, prepared by Hyland Laboratories, Los Angeles, were collected and pooled from persons of blood group B and A respectively. The titer of the undiluted sera by the method previously described (15) was 1:64 - 1:128 for the anti-A serum and 1:256 for the anti-B serum. The suspension of hog lining was shaken continuously for about one hour at room temperature (about 25°). Then the insoluble matter was centrifuged off, the supernatant decanted, and 0.5 ml of a 1% suspension of the appropriate blood cells was added to the supernatant. The cells, obtained from Hyland Laboratories, were pools of at least six different donors. After standing for one-half hour at 25° the cell suspensions were centrifuged for one minute and then read micro-

scopically for agglutination (15). Results are shown in Table IV. "0" indicates no agglutination of cells; "+", a few two-celled clumps; "+++" to "++++", clumps of many cells; and "+++++", complete agglutination with only a few unagglutinated cells visible in the microscopic field. Control 1 consisted of 0.5 ml of saline, 0.5 ml of serum, and 0.5 ml of 1% cell suspension. Control 2, which consisted of 1 ml saline and 0.5 ml of 1% cell suspension, indicates the absence of agglutination of cells due to non-specific factors.

Isolation of A-substance from hog stomach lining by extraction with dilute hydrochloric acid. A mixture of 5.1 g of hog 5 stomach lining, which possessed A-substance, and 100 ml of 0.15 M HCl was shaken for one hour. Since the solution was quite viscous, 200 ml of H_2O were added and the solution centrifuged. The solution did not centrifuge readily and only a fraction of the total centrifugates was reserved. Then to the centrifugate was added about one gram of sodium acetate and two volumes of 95% ethanol. The precipitate obtained was suspended in 100 ml of 0.5% sodium acetate solution and undissolved material centrifuged off and discarded. A small amount of precipitate was obtained when two volumes of alcohol were added to the centrifugate. The precipitate was dissolved in 20 ml of water, dialyzed overnight in the cold room and then lyophilized. Solid amounting to 47 mg was recovered (H5A-F1). This fraction had a relative A activity of 1 in the isoagglutination inhibition and the hemolysis inhibition tests.

Discussion

Table II shows that of twelve individual hog stomach linings which were studied only four, hogs 2, 3, 5 and 6, contained A-substance. Witebsky (29) found only 40% of hogs to have the A specific factor while Bendich, Kabat, and Bezer (3) found seven A active stomachs out of ten tested and Aminoff, Morgan, and Bray (1) found fourteen out of twenty-four stomachs to contain the A factor. The other eight inactive preparations, presumably containing O-substance, are similar to the A active ones in appearance and yield. All the preparations were obtained in yields varying from 2% to 10% from the dried hog stomach linings. Since the linings weighed about 30 grams, 0.5 - 3 grams of the preparations were usually isolated. The yield and activity of these preparations emphasizes one of the original advantages of the use of mucin as a starting material since it is calculated that the one kilogram of mucin used as starting material in Part IV represents the yield of A active material from approximately 200 to 300 hog stomach linings.

The A specific preparations are only about 30 - 60% as active as the most active fractions obtained from hog mucin (Part IV and Part V). This would indicate that these preparations are impure, as was suggested by evidence from absorption spectra discussed in Part III. The mere absence of O-substance would not then appear to be obigatory for obtaining highly potent preparations. Furthermore, unless fractionation is possible with hog stomach linings far in excess with that possible with mucin, the relative activities suggest

Table II

Serological Properties of Preparations Isolated from Peptic Digests of Individual Hog Stomach Linings

Hog No.	Description of fraction	Fraction	Yield %	Relative A activity	
				Hemolysis test	Isoagglutination test
1	Insoluble 3 vol. ethanol.	H1-F3	7.4	< 0.0015	< 0.002
	Residue after digestion.	H1-R	26		
2	Insoluble 2 vol. ethanol.	H2-F1	3.0	3.5	3
	Soluble 2 vol. ethanol; insoluble 3 1/2 vol. ethanol.	H2-F3	4.3	.5	.03
	Residue after digestion.	H2-R	20		
3	Insoluble 2 vol. ethanol.	H3-F1	9.2	2	2.5
	Soluble 2 vol. ethanol; insoluble 3 vol. ethanol.	H3-F2	5.0	.4	.07
	Residue after digestion.	H3-R	15		
4	Insoluble 3 vol. ethanol.	H4-F1	12.0	< .0015	< .002
	Residue after digestion.	H4-R	15		
5	Insoluble 3 vol. ethanol.	H5-F1	7.1	1	.5
	Residue after digestion.	H5-R	23		
6	Insoluble 3 vol. ethanol.	H6-F1	7.4	3	1.5
	Residue after digestion.	H6-R	19		
7	Insoluble 3 vol. ethanol.	H7-F1	4.5	.0015	< .0007
	Residue after digestion.	H7-R	30		
8	Insoluble 3 vol. ethanol.	H8-F1	7.5	< .0008	< .0007
	Residue after digestion.	H8-R	26		
9	Insoluble 3 vol. ethanol.	H9-F1	5.2	< .0008	< .0007
	Residue after digestion.	H9-R	19		
10	Insoluble 3 vol. ethanol.	H10-F1	1.9	.0015	< .0007
	Residue after digestion.	H10-R	27		
11	Insoluble 3 vol. ethanol.	H11-F1	2.8	.001	< .0007
	Residue after digestion.	H11-R	32		
12	Insoluble 3 vol. ethanol.	H12-F1	6.2	.001	< .001
	Residue after digestion.	H12-R	21		

that A-substance from mucin possesses little O-substance on a weight basis.

At least two fractions have been distinguished in the material soluble in water and insoluble on addition of three volumes of ethanol: one is insoluble on addition of two volumes of alcohol (H2-F1 and H3-F1), while the other is soluble on addition of two volumes of alcohol but insoluble on addition of three volumes (H2-F3 and H3-F2). H2-F1 and H3-F1 are considerably more active than H2-F3 and H3-F2; this result is in agreement with observations on mucin where the active fractions are concentrated in fractions insoluble in 60% ethanol. However, fractions corresponding to H2-F3 and H3-F2 have not been detected in hog gastric mucin. Hence, fractionation procedures which are suitable for mucin must be applied with caution to the isolation of material from digested hog linings. Furthermore, the relative activities of the fractions soluble in 66% ethanol suggest that A-substance has been degraded. The relative activities of these fractions in the hemolysis test is 7 - 16 times greater than in the isoagglutination test, a result similar to that observed for the degraded preparations from mucin (4). Undegraded preparations from mucin have approximately equivalent relative activities in these tests. The degradation of A-substance in pepsin has been previously recognized (4) but the possibility of its occurrence in peptic digests of hog stomach linings has not been realized. As has been discussed in Part V for a similar situation, the existence of the two forms of A-substance in peptic digests, differing as to degradation, introduces uncertainty regarding the general applicability

of the precipitin test of Bendich, Kabat, and Bezer (3) for determining absolute purity since the method assumes that a single substance is involved in the antigen-antibody precipitate.

The results obtained with inactive preparations indicate that the hemolysis inhibition test is actually fairly specific as a test for A-substance. Despite the fact that the anti-human A cell rabbit immune sera agglutinates human O cells (44) very little inhibition of hemolysis of sheep cells is observed for these preparations. This is in agreement with other observations that Forssman antigen-like properties are confined to the blood group A-substance (46). It should be noted, however, that a detectable and in some cases measurable inhibition occurs for the inactive preparations in both the hemolysis and the isoagglutination tests. The small quantity of A-substance present in the Plaut crystalline pepsin is insufficient to account for this inhibition. The nature of this cross-reaction is not understood.

The equivalent N-acetyl glucosamine content (15) of some of the preparations from hog linings have been determined and results are given in Table III. The A specific fractions, H2-F1 and H3-F1 show 4-7% equivalent N-acetyl glucosamine, values which are much lower than those found for preparations of similar activity from hog mucin. Fractions H2-F3 and H3-F1, which have considerably less activity, show correspondingly less N-acetyl glucosamine. It is not known whether the low N-acetyl glucosamine contents found for these preparations compared to preparations from hog mucin are due to some difference in chemical structure or to some of the factors, such as

presence of buffer, or alkali-neutralizing material, which are known to affect the absolute amount of N-acetyl glucosamine detectable (15). The fractions, H1-F3 and H4-F1, which have no A specific properties, also possess equivalent N-acetyl glucosamine to the extent of 3%. This would suggest that probably the O-substance from hog stomach, presumably in these fractions, also gives the color reaction with Ehrlich's reagent. It has been previously observed that A, O, and B substances from human ovarian cyst fluids (38), AB substance from horse stomach (15), and A substance from human erythrocytes (15) give similar color tests. These results support the hypothesis that the

Table III

Fractions	Equivalent N-acetyl glucosamine content %	Relative A activity hemolysis test
H1-F3	3	< 0.0015
H2-F1	7	3.5
H2-F3	0	.5
H3-F1	4	2
H3-F2	0	.4
H4-F1	3	< .0015

groupings responsible for the color reaction are a common feature of the haptens having blood group O, A, or B properties. The correlation

of A activity and the N-acetyl glucosamine content reported earlier (4, 15) for preparations from hog gastric mucin may now be presumed to be subject to deviations in case the fractionation has involved the separation of A and O substance, both of which have N-acetyl glucosamine detectable by the test with Ehrlich's reagent. Such deviations have in fact been observed in the correlation of the color test with the more precise hemolysis test (4).

The existence of either A or O substance in hog stomach has presented the problem of the detection of these materials conveniently. At present the detection is presumably not possible before several days of enzymatic digestion and subsequent isolation (3). The early detection of A or O substance is important not only from the standpoint of the practical problem of accumulating efficiently either one or the other substance but also because evidence discussed above suggests that the pepsin digestion probably gives rise to a degraded product. As a result a rapid method for the detection of A-substance in individual hog stomach linings has been devised which uses very little material and which can be applied prior to any preparative work. The method involves the adsorption technique (46) which has been used frequently for the detection of haptenic components in tissues. The detection of A-substance by this means is demonstrated by the results in Table IV. Dried hog lining is incubated with anti-A serum for one hour and the supernatant tested for agglutination of human blood group A cells. Inhibition of agglutination indicates the presence of A-substance. If too little serum is used non-specific adsorption may interfere but as the results in Table IV show 100 microliters of

Table IV

Inhibition of Isoagglutination of Human Blood Cells after Preliminary Incubation of Serum with Hog Stomach Linings

Hog No.	A cells: 20 µl anti-A serum	A cells: 100 µl anti-A serum	B cells: 20 µl anti-B serum	A activity of fractions isolated from lining by peptic digestion
Control 1 (serum +cells)	++++	+++++	++++	
Control 2 (saline +cells)	0	0	0	
1	+	++++		Inactive
2	0	0	++++	Active
3	0	0	++++	Active
4	+	++++		Inactive
5	0	0	+++	Active
6	0	0	+++	Active
7	+	+++		Inactive
8	+	++++		Inactive
9	+++	++++		Inactive
10	+	++++		Inactive
11	+++	++++		Inactive
12	0	+++		Inactive

anti-A serum incubated with 10 mg of hog lining was sufficient to detect A-substance unequivocally in hogs 2, 3, 5 and 6. The absence of inhibition of agglutination of B cells by a small amount of anti-B serum confirms that the inhibition of A cell agglutination was specific.

The detection of A-substance by the above experiments elicits a question with regard to its mode of combination in hog stomach linings. Bendich, Kabat, and Bezer (3) could not obtain active A-substance from dried stomach lining by extraction with 90% phenol. A peptide combination was suggested since trypsin digestion yielded active material, although in small yields, while peptic digestion or autolysis liberated large amounts of active fractions. Tryptic and peptic digestion have also been used previously in commercial preparations (Lilly and Sharpe and Dohme). However the preparation of mucin containing A-substance by the procedure of Fogelson (31, 45) involves essentially an acid extraction without apparently any enzymatic digestion. Since water-soluble A-substance can be derived from this source by non-enzymatic methods, it seems likely that at least a part of the A-substance is loosely bound and dispersable in acid. A confirmation of this hypothesis and proof that preliminary enzymatic digestion was not obligatory to the liberation of water-soluble A-substance was obtained by subjecting an acid extract of hog stomach lining to several alcohol fractionations. A water-soluble fraction, H5A-F1, was obtained which had the same activity as the fraction obtained by peptic digestion, H5-F1. This observation opens the possibility of investigating the isolation of A-substance from

hog linings by mild procedures which circumvent the degradation occurring on peptic digestion. In this connection it is significant that H5A-F1, prepared by acid extraction, differs substantially in properties from H5-F1 since the former yields insoluble material on addition of acid (Part V).

Summary

Partially purified A-substance preparations from individual hog stomach linings have been isolated by peptic digestion. These preparations, compared to those from mucin, 1) appear to be slightly degraded 2) have less equivalent N-acetyl glucosamine, and 3) are only 30 - 60% as potent in inhibition of hemolysis and isoagglutination tests.

A simple method for the detection of A-substance in hog stomach linings is described and the significance of the test is discussed.

VII Observations on the Alkaline Degradation of Blood Group A Substance from Hog Gastric Mucin

In this section some observations are reported with regard to the degradation of A-substance from hog gastric mucin with alkali. The degradation with alkali is of interest because of the unusual susceptibility of A-substance to this reagent and because degradation under these condition yields material giving a color test with Ehrlich's reagent (p-dimethylaminobenzaldehyde). The latter test provides an expedient method for studying the isolation of degradation products. Since this study was initiated when only partially purified A-substance preparations were available, many of the observations must be regarded mainly as orienting for further work on more purified preparations. However, in as much as the products giving the test with Ehrlich's reagent have been principally studied and since the colors obtained with this reagent are indicative of blood group properties (4, 15, 37), observations on these moieties are of interest even though the starting materials were impure.

In 1934 Freudenberg and Eichel (57; 58) reported the ready lability of A-substance from human urine to alkali. Loss of acetyl occurred on digestion with alkali and a loss of activity in a hemolysis inhibition test occurred concomitantly. The loss in activity was presumed to be due to the loss of acetyl since the activity was completely restored on reacetylation. Evidence for the decomposition of A-substance with alkali is apparent from the results of Gróh, Szélyes, and Weltner (9) and by Weltner (30) on the ultraviolet absorption spectra of A-substance from human urine and from hog stomachs. It was found that the extinction values of A-substance solutions increased steadily in the presence of alkali suggesting decomposition

reactions. Further evidence for a more far-reaching alteration in structure was presented by Morgan and King (24). It was shown that on mild treatment with alkali, A-substance from hog gastric mucin decreased in potency in the isoagglutination inhibition test, while the activity in the hemolysis inhibition test was unaltered or enhanced. Recently Kabat, Bendich and Bezer (18) have shown that alkali treatment causes a decrease in potency in a precipitation test with anti-A serum (34). In addition, in connection with observations on Ehrlich's test, which involves a preliminary treatment with alkali, Morgan and King (24) reported that the reducing properties (copper reduction) as glucose of A-substance increased from 0.4-0.6% to 3.3% after treatment with alkali. At the same time 12-13% N-acetyl glucosamine was detected with Ehrlich's reagent (36). The reducing properties after alkaline digestion corresponded to the same amount of N-acetyl glucosamine when it was found that with the copper reduction method used N-acetyl glucosamine is equivalent to only 27% of an equal weight of glucose. These results led to the conclusion that the action of alkali was to break a labile glycosidic link at carbon atom one of N-acetyl glucosamine in A-substance; the liberated N-acetyl glucosamine group then underwent subsequent reactions to give Ehrlich's test. The reducing group of N-acetyl glucosamine is required since N-acetyl and N-benzoylmethyl glucosaminides do not give Ehrlich's test. In a recent note Morgan (59) has reported that the product giving Ehrlich's test is dialyzable.

The product from N-acetyl glucosamine which is derived on treatment with alkali has been shown to be 2 methyl- Δ^2-glucoxazoline by

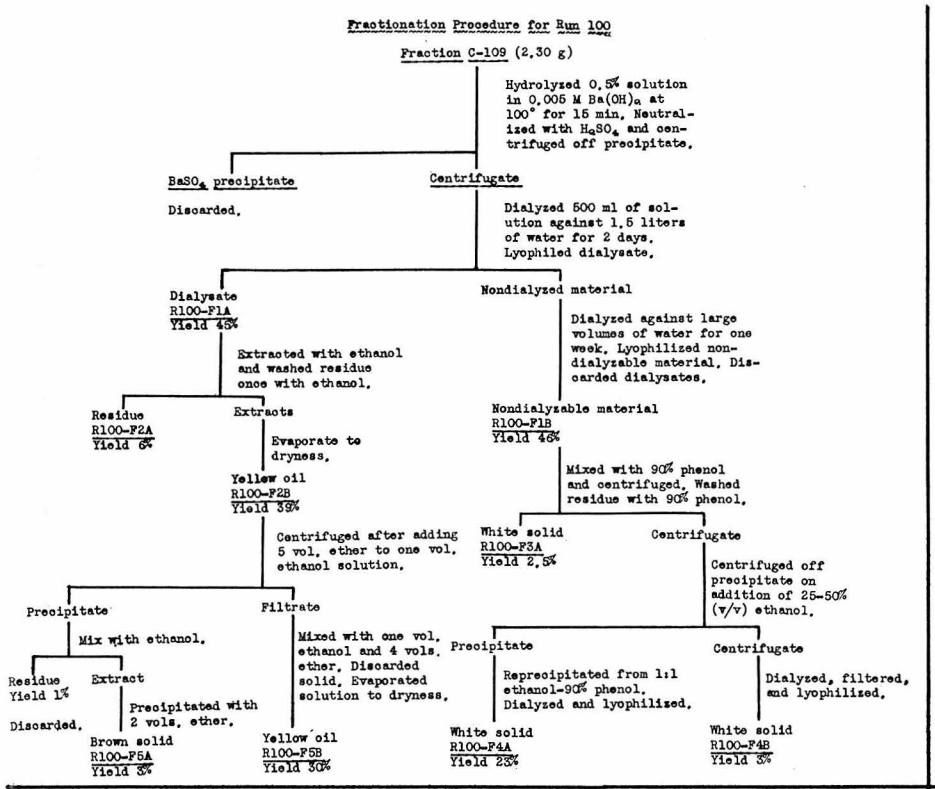
White (60). By analogy the same or similar reaction product is presumed to occur in the degradation of A-substance with alkali. However, it is shown in this section that several anomalies exist in the properties of the reaction products from N-acetyl glucosamine and A-substance which are not in agreement with White's structure.

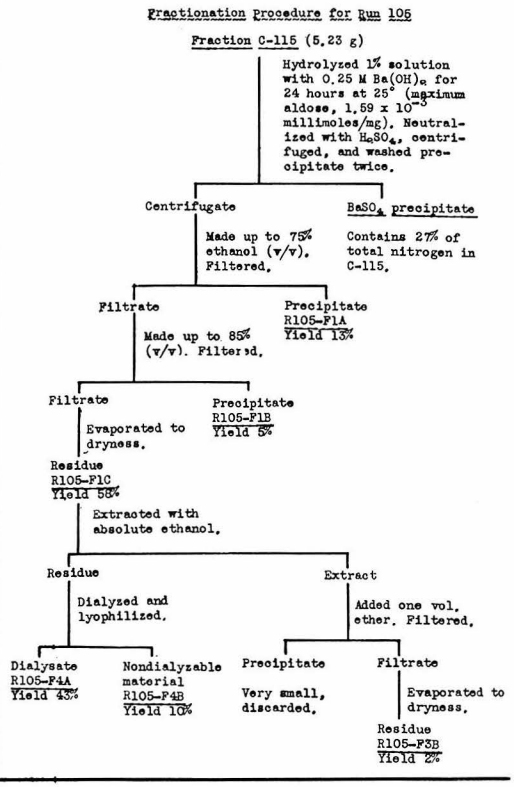
Experimental

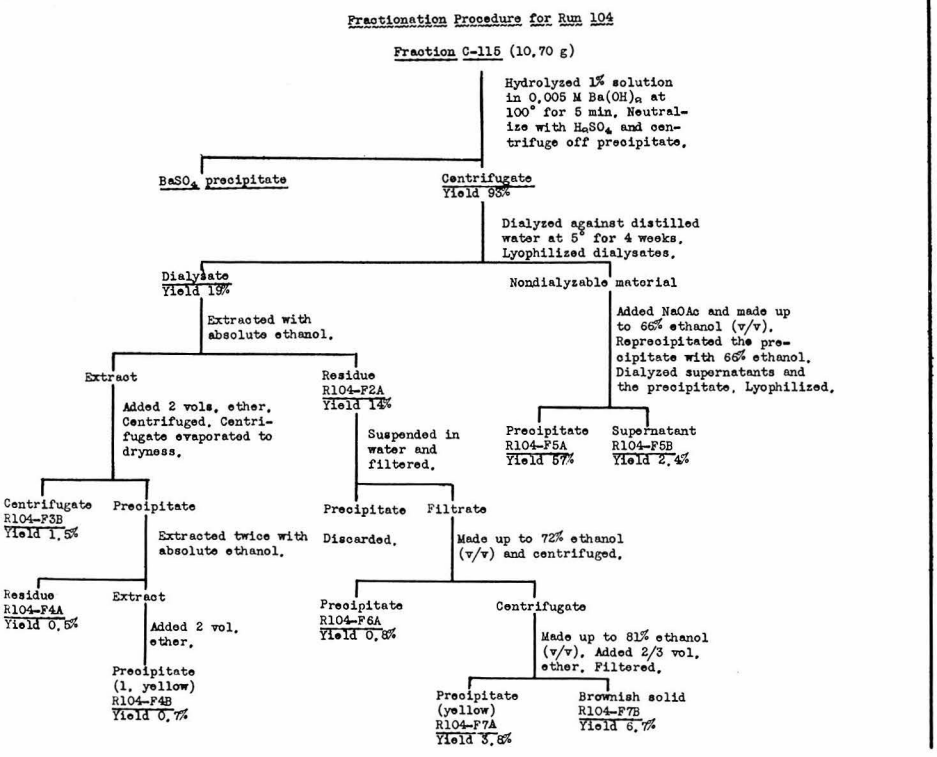
Alkaline degradation of A-substance from hog mucin. Outlines of fractionation procedures for the separation of components in alkaline degradation mixtures of A-substance are shown in Fig. 1. The A-substance used as a starting material in these procedures, C-109 and C-115, are fractions obtained from hog gastric mucin, soluble in water, soluble in 30% ethanol, and insoluble in 45% ethanol (see Part II). The activities of these fractions are not high, being only about the same as that of the total water-soluble solids in mucin. The isolation of degradation products of A-substance from concentrated barium hydroxide solutions (R105 and R106) is unsatisfactory since the removal of barium as sulfate or carbonate is accompanied by considerable losses of total nitrogen. Runs R100 and R104 are more satisfactory since the barium precipitates are small.

Fraction R1-F2A (see Part II) which was used in the study of the rate of degradation (Tables I and II) was considerably more active than the above fractions having an activity in the inhibition of hemolysis test of about three times that of C-109 and C-115.

Alkaline degradation of N-acetyl glucosamine. The conditions of White (60) were used for the production of 2-methyl-Δ^2-glucoxazoline from N-acetyl glucosamine. The conditions of fractionation were modified in as much as dark brown products could be largely removed by







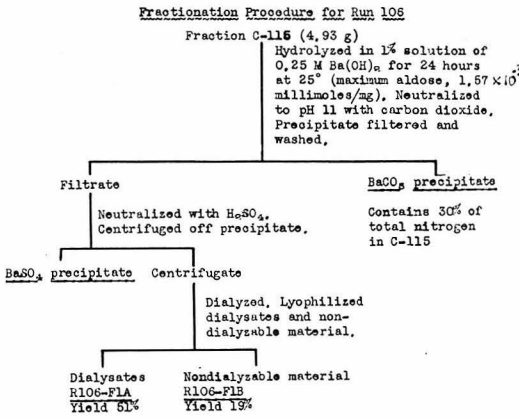


FIG. 1 . OUTLINES OF FRACTIONATION PROCEDURES

fractionating from alcohol and ether solutions.

A 0.46% solution of N-acetyl glucosamine (1.5 liters), 0.02 M NaOH, was heated at 63-64° for one hour. The solution had a bright yellow color. The solution was cooled, neutralized with hydrochloric acid and then lyophilized. The solid was extracted five times with 50 ml portions of absolute ethanol; the residue which gave a weak Ehrlich's test was discarded. The extracts were evaporated to about 100 ml, yielding a dark red-brown solution. A very dark brown oil was obtained on adding one volume of ether and more precipitate was obtained on adding up to 900 ml of ether. The total precipitate which yielded a brown oil on filtering amounted to 44% and was less effective in giving Ehrlich's test than the filtrate. The filtrate was evaporated to dryness, 20 ml methanol added, and the brown precipitate separating after adding four volumes of ether (fraction F6A) was reserved (yield 6%). The ether-methanol solution was evaporated to a small volume, 50 volumes of ether added and the light yellow oil separated and dried (fraction F6B, yield 11%); the oil solidified on drying.

Fractions F6A and F6B were very hygroscopic and gave intense colors with Ehrlich's reagent without any preliminary treatment with alkali. Fraction F6B gave 155% equivalent N-acetyl glucosamine directly and fraction F6A gave 120%. Fraction F6B contained 6.66% nitrogen (Kjeldahl) and fraction F6A, 6.53%. (Theory for 2-methyl-Δ^2-glucoxazoline, 6.90%).

Results and Discussion

1. Nature of the Alkaline Decomposition of A-substance

In Table I are shown results of a study of the rate of alkaline degradation of A-substance at 100°. The conditions are the same as those used in the preliminary digestion of A-substance in the test with Ehrlich's reagent (4), namely, 0.1 ml of 0.25 M Na_2CO_3 per ml of solution. It is seen that the maximum equivalent N-acetyl glucosamine content[1] is attained in 10 to 15 minutes. The decrease in equivalent N-acetyl glucosamine is presumably due to degradation reactions; the accumulation of degradation products is evidenced by the continual increase in intensity of the yellow color of the solution on digestion (see Klett readings for the solution with a blue filter, column 3, Table 1). Degradation is also apparent from the decrease in the pH of the solution from 10.5 to 9.3 after 4 hours of hydrolysis. The decrease in pH could be due to deacetylation; it was found in 0.02 M NaOH that about 25% of the acetyl of N-acetyl glucosamine was hydrolyzed in 30 minutes at 76° on the basis of alkali consumption. The production of reducing material on alkaline treatment is shown by the marked increase in aldose from 0.38×10^{-3} to 1.19×10^{-3} moles per gram after five minutes digestion. The aldose content is measured by oxidation of free aldehyde groups with hypoiodite to carboxyl groups (61)[2]. The maximum aldose is produced

---

1 The equivalent N-acetyl glucosamine content was obtained by comparing the colors obtained with Ehrlich's reagent with those obtained for various amounts of N-acetyl glucosamine (see Part I).

2 The aldose content of the fraction before hydrolysis may not be due to aldehyde groups since it was shown that few carboxyl groups were produced after hypoiodite oxidation by means of acid-base titrations. Possibly reaction with other reducing materials could account for the initial aldose, e.g., arginine, histidine, and tyrosine.

Table I

Alkaline Degradation[1] of A-substance (R1-F2A) at 100°

Time of hydrolysis (minutes)	pH	Klett reading (blue filter)	Equivalent N-acetyl glucosamine (%)	Aldose (moles x 10^3 per gram)	Percent nondialyzable material
0				0.38	100
5	10.5	27	10	1.19	79
10	10.5	50	13	1.35	69
15	10.4	86	13	1.80	62
30	10.1	127	12	2.00	49
60	9.8	187	8	2.19	38
120	9.5	260	6	2.20	32
240	9.3	320	4	2.15	20

1 Conditions: 0.1 ml of 0.25 M Na_2CO_3 per ml of ca 0.1 % solution in ampoules.

after approximately one hour of hydrolysis and corresponds approximately to 40% glucose. The extensive decomposition of A-substance is also indicated by the amount of material which becomes dialyzable after treatment. The dialyzable fraction increases steadily until after four hours hydrolysis, 80% of the added A-substance preparation is dialyzable.

The degradation of A-substance occurs rapidly at 100° in alkali and considerably slower at lower temperatures. In Table II are given results for the degradation of A-substance in 0.92 M NaOH at 25°. The reaction is much slower than at 100° as may be seen from the fact that 43 hours are required in much more alkaline solution for the attainment of the maximum apparent aldose value. However, the reaction at 25° is substantially different than at 100° even though approximately the same aldose content was attained in both cases. Thus, very little apparent equivalent N-acetyl glucosamine was detectable with Ehrlich's test without heating; a maximum of 2% equivalent N-acetyl glucosamine was attained after about 10 hours. Considerably larger amounts of N-acetyl glucosamine were detected if the solutions were heated[1]; a maximum of 10% was attained on 5 minutes heating after 4-5 hours hydrolysis while the maximum N-acetyl glucosamine detectable on 15 minutes heating, 12%, decreases steadily on hydrolysis. It would appear that these results could be interpreted as involving simply the breaking of the alkali-labile linkage of A-substance

---

[1] The procedure used was as follows: the sodium hydroxide solutions were neutralized with hydrochloric acid and 1 ml aliquots heated with 0.1 ml of 0.25 M Na_2CO_3 after the conditions of Ehrlich's test (Part I).

Table II

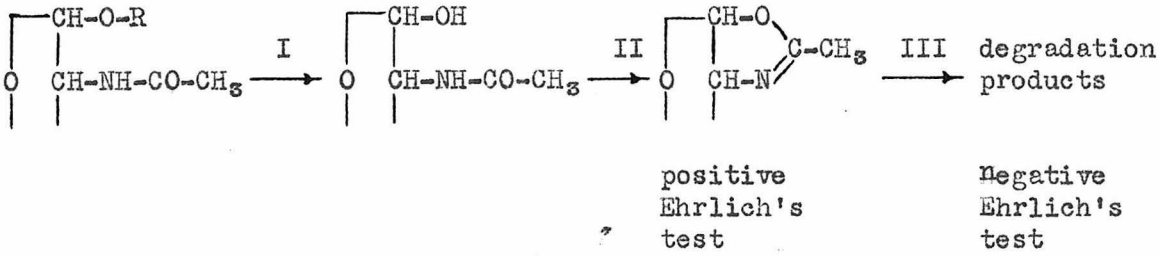
Alkaline Degradation[1] of A-substance (R1-F2A) at 25°

Time of hydrolysis (hours)	Aldose (moles x 10^3 per gram)	Equivalent N-acetyl glucosamine (%)		
		No heating	5 min. heating	15 min. heating
0	0.38	0	5	12
.53	.58	0	7	12
1.42	.79	0	7	11
2.45	.93	0	8	11
4.24	1.24	1	10	11
5.40	1.30	2	10	10
14.6	1.91	2	11	8
40.0		1	8	5
43.2	2.20			
64.7	2.18			

1 Conditions: 0.92 M NaOH, 0.8% solution; aliquots neutralized before analysis.

without subsequent appreciable reaction at 25° to give the product reacting with Ehrlich's reagent. This is supported by the fact that after long hydrolysis the maximum N-acetyl glucosamine is attained on heating 5 minutes rather than 15 minutes. It has been previously observed (4) that the maximum N-acetyl glucosamine content is attained for unhydrolyzed A-substance in 15 minutes; N-acetyl glucosamine which contains a free aldehyde group attains a maximum value in only 5 minutes (36).

The results in Tables I and II offer evidence in support of the following series of reactions occurring on degradation of A-substance with alkali. A-substance with the alkali-labile linkage attached to R^1



is broken at 25° in the cold (Reaction I); this is supported by the increase in aldose content[2]. The subsequent reaction of the N-acetyl

---

[1] Morgan and Van Heyningen (37) and Bray, Henry, and Stacey (2) assumed that the R group may be an amino acid due to its ready splitting by alkali; a glycosidic sugar linkage would presumably be stable under these conditions.

[2] N-acetyl glucosamine reacts practically stoichiometrically with hypoiodite while the sugar heated with sodium carbonate gives results only 10-15% high. Since the increase in aldose content on hydrolysis of A-substance is greater than can be accounted for by the maximum N-acetyl glucosamine detectable by Ehrlich's reagent, other reactions leading to the reducing substances must be involved. The suggestion of Morgan and King (24) regarding the equivalence of N-acetyl glucosamine and reducing power after hydrolysis of A-substance is based on results with a non-stoichiometric copper reduction method.

glucosamine group leading to the product giving Ehrlich's test (Reaction II) should be similar to that of N-acetyl glucosamine itself. Thus when appreciable hydrolysis has occurred the maximum color in Ehrlich's test is attained in 5 minutes heating as occurs for N-acetyl glucosamine. At 100° Reactions I and II proceed succesively at comparable velocities. Since the color with Ehrlich's reagent decreases on prolonged heating (Table I), it must be assumed that a reaction (III) leads to degradation. It is possible, of course, that degradation as judged by Ehrlich's test could also occur by deacetylation of either of the components in Reaction I.

It is emphasized that the series of reactions postulated are based on the assumption that N-acetyl glucosamine in A-substance is in fact the group responsible for the test with Ehrlich's reagent. Evidence for the similarity of the products giving Ehrlich's test in A-substance and N-acetyl glucosamine is discussed below. It should be pointed out, however, that the structure which is presumed to give the test with Ehrlich's reagent, 2-methyl-Δ^2-glucoxazoline, appears questionable and is discussed below; the particular series of reactions mentioned above do not depend on any assumption regarding the validity of this structure.

2. Nature of Products Isolated on Alkaline Decomposition of A-substance

A-substance has been degraded with alkali under various conditions and the mixtures fractionated by dialysis and precipitations with organic solvents (see Fig. 1). In Table III the immunological properties of various fractions are summarized. Dialyzable fractions have no

Table III

Immunological Properties of Alkaline Degradation Products of A-substance Preparations

Description of fraction	Fraction	Relative A activity[1]	
		Isoagglutination inhibition test	Hemolysis inhibition test
Starting material	C-109	1.1	1.5
	C-115	1.2	1.5
Dialyzable	R100-F1A	<.006	
	R100-F2A	<.006	
	R100-F2B	<.004	<.02
Nondialyzable	R100-F1B	.02	
	R100-F3A	.08	
	R100-F4A	.007	.2
	R100-F4B	<.007	
	R104-F5A	.1	.9
Dialyzable	R105-F4A		.0004
Nondialyzable	R105-F1A[2]	.01	.1
	R105-F1B[2]	<.001	.02

1 Wilson hog gastric mucin assumed to have unit activity.
2 Nondialyzable concluded from fractionation procedure, i.e., low solubility in alcohol-water mixtures.

activity in the isoagglutination inhibition or hemolysis inhibition tests. Nondialyzable fractions retain some potency towards both tests. In every instance, however, the potency in the hemolysis inhibition test is 10-20 times greater than in the isoagglutination inhibition test, an observation which is in agreement with previous observations on degraded preparations from mucin (15, 19, 24). Prolonged action of alkali, however, decreases the potency of the product in the hemolysis inhibition test also; thus considerably less activity occurs in the nondialyzable fractions of Run 100 (15 minutes heating at 100°) as compared with Run 104 (5 minutes heating at 100°). The diminution in potency on prolonged treatment with alkali recalls experiments of Freudenberg and Eichel (58) in which the potency in the inhibition of hemolysis test was destroyed after 6 hours hydrolysis of A-substance from human urine in saturated barium hydroxide at 100°[1].

In Table IV some of the chemical properties of fractions obtained by alkaline degradation are summarized. It is apparent from these results that the products giving a color with Ehrlich's reagent are low enough molecular weight to be dialyzable[2]. The nondialyzable fractions give very little or no color directly, but show low equivalent N-acetyl glucosamine contents after heating 15 minutes with alkali under the conditions of the color test (4). The mildest treat-

---

[1] It has been found that 6 hours treatment of A-substance from hog gastric mucin with 0.25M $Ba(OH)_2$ at 100° resulted in over 99% of the material being dialyzable. No experiments have been tried to test the remarkable reappearance of full potency after this degradation by reacetylation as has been described by Freudenberg and Eichel (58).

[2] Visking cellulose sausage casing was used in this and other experiments.

Table IV

Chemical Properties of Alkaline Degradation

Products of A-substance Preparations

Description of fraction	Fraction	Total N (%)	Aldose (moles x 10^3 per gram)	Equivalent galactose (%)	Equivalent N-acetyl glucosamine (%) No heating	Equivalent N-acetyl glucosamine (%) 15 min. heating
Starting material	C-109	6.8	0.26		0	11
	C-115	7.0			0	11
Dialyzable	R100-F1A	2.4	1.7	15	13	8
	R100-F2A		1.4	21	7	4
	R100-F2B	1.6	1.4	13	13	7
Nondialyzable	R100-F1B	7.6	1.1	26	2	6
	R100-F3A		.4	33	0	2
	R100-F4A	6.1	1.1	27	0	6
	R100-F4B		1.6	19	0	3
Dialyzable	R104-F2A	7.2	1.7		29	
	R104-F3B	4.1	3.8		38	
	R104-F4A	5.0	1.7		47	
	R104-F4B	4.7	2.1		63	
	R104-F6A	10.1	.3		3	
	R104-F7A	7.7	.6		9	
	R104-F7B	5.1	2.4		44	
Nondialyzable	R104-F5A	6.3	.4		0	
	R104-F5B	7.1	.9		.5	
Dialyzable	R105-F4A	4.2	1.7		13	9
Nondialyzable	R105-F1A	7.5	1.2		0	5
	R105-F1B	7.2	1.2		1	4
	R105-F4B	7.7	.2		1	4
Dialyzable	R106-F1A	3.8	1.9		12	9
Nondialyzable	R106-F1B	7.4	1.4		0	4

ment with alkali, Run R104, yielded fractions with very high equivalent N-acetyl glucosamine content, for example, R104-F4B contains 63%. The fractions giving Ehrlich's test directly, show less color on further treatment with alkali in agreement with qualitative observations above (Reaction III). The majority of the reducing power of the degradation products occurs in the dialyzable fractions. It appears also that the dialyzable fractions have significantly less total nitrogen than the nondialyzable fractions. All the fractions (R100) show hexose by the procedure of Gurin and Hood (33). Considerable improvement in the purity of the fractions giving Ehrlich's test should be possible.

In Part IV it was pointed out that the visible spectra of the colors obtained with Ehrlich's reagent for A-substance and N-acetyl glucosamine were identical. Another point of similarity is derived from the ultraviolet absorption spectra of degradation products. In Fig. 2 the extinction curves of products obtained on alkaline degradation of A-substance are shown. The dialyzable fraction, R104-F1A, accounts for the increase in extinction in the ultraviolet occurring on alkaline degradation; the nondialyzable fractions, R104-F5A and R104-F5B, show diminished extinction in the ultraviolet compared with the parent compound, C-115. It would appear that the observation of Weltner (30) as to the increase in the extinction in the ultraviolet is due to the formation of the dialyzable fractions. When the dialyzable fraction, R104-F1A, was fractionated yielding among others, fractions R104-F4B, R104-F7A, and R104-F7B, considerable differentiation in the ultraviolet spectra are apparent. Fractions R104-F7B and

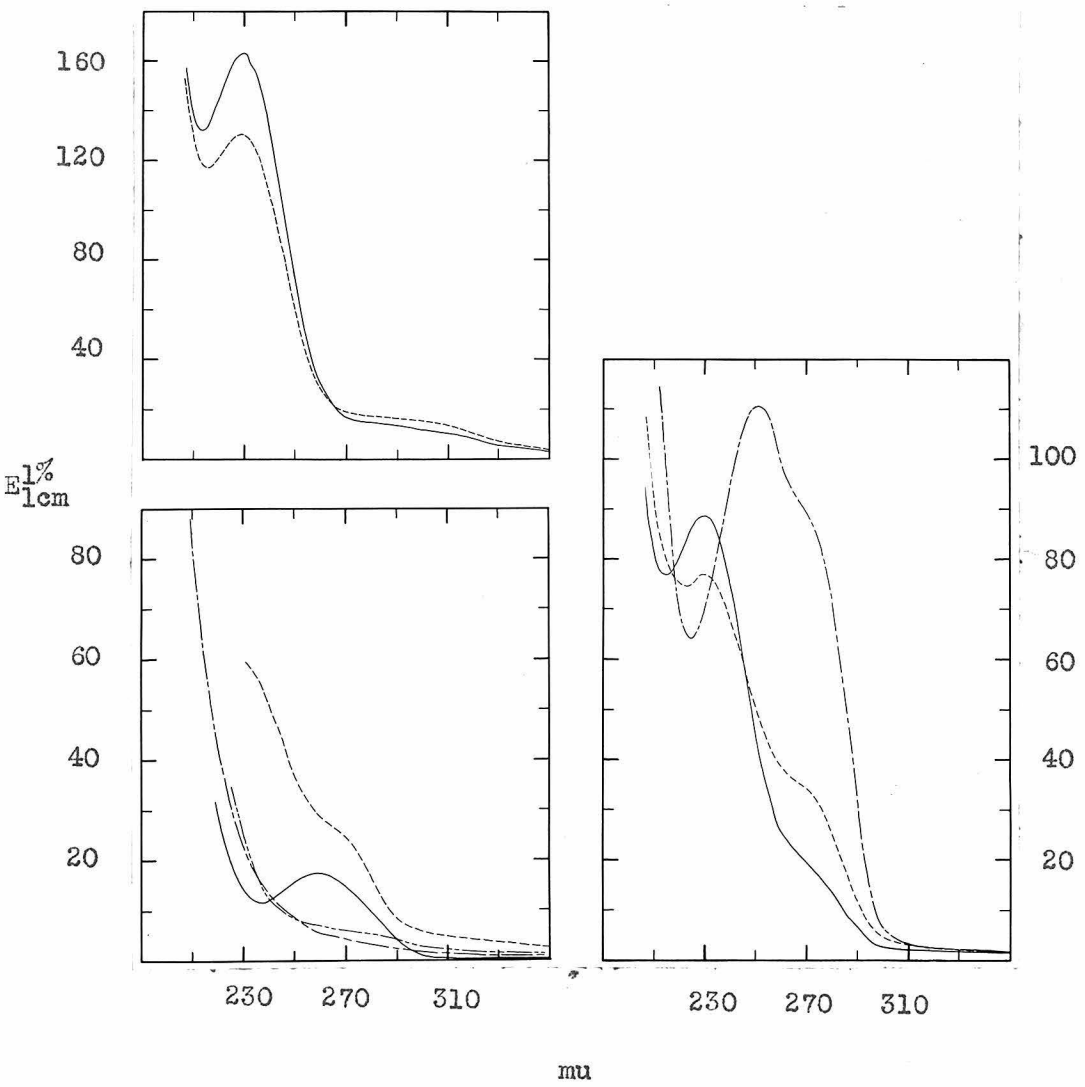


Fig. 2 . Upper left: Extinction curves of fractions isolated from alkaline digestion of N-acetyl glucosamine. ———, fraction F-6B; – – – –, fraction F-6A.

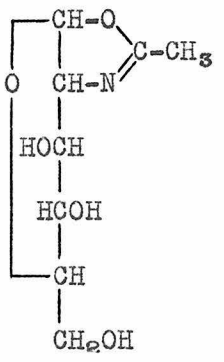
Lower left: Extinction curves of fractions isolated from alkaline digestion of partially purified A-substance from hog mucin. – – – –, R104-F1A; ———, C-115; – – – –, R104-F5B; — – —, R104-F5A.

Lower right: Extinction curves of fractions isolated from alkaline digestion of partially purified A-substance from hog mucin. ———, R104-F4B; · · · · · · · ·, R104-F7B; — – —, R104-F7A.

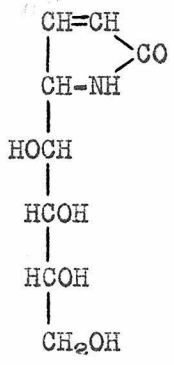
R104-F4B, which have respectively, 44% and 63% equivalent N-acetyl glucosamine, have extinction curves with maxima at 230 mu, $E^{1\%}_{1cm}$ 77 and 89, respectively. Fraction R104-F7A, which shows less equivalent N-acetyl glucosamine, 9%, has no peak at 230 mu but shows one at 250 mu. The peak at 230 mu appears characteristic for the products giving Ehrlich's color test, thus the products from the alkaline digestion of N-acetyl glucosamine, fractions F6A and F6B, which have, respectively, 120% and 155% equivalent N-acetyl glucosamine, shows correspondingly higher peaks at 230 mu, $E^{1\%}_{1cm}$ 130 and 163.

3. A Criticism of White's Structure of 2-methyl-Δ^2-glucoxazoline

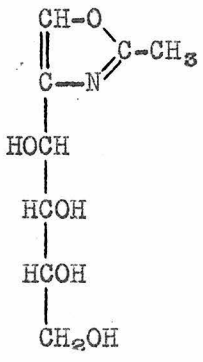
In 1940 White (60) presented evidence for favoring 2-methyl-4', 5'-glucopyrano-Δ^2-oxazoline (A) as the structure for the reaction product of N-acetyl glucosamine with alkali. By analogy, Bromund and Herbst (62) suggested the same structure for several other acyl derivatives of glucosamine. A pyrroline (B) type structure, suggested by Zuckerkandl and Messiner-Klebermass (63) was eliminated by Morgan (64) due to the fact that N-benzoyl and N-trimethylacetyl glucosamine, in which no hydrogen on the alpha carbon was present,



(A)

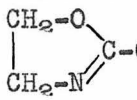


(B)



(C)

gave positive Ehrlich's tests. A diglucopyrazine stucture produced by condensation of two moles of N-acetyl glucosamine was possible since N-acetylaminoacetaldehyde condensed to form N-diacetyldihydropyrazine (64) which gave a positive Ehrlich's test. This structure was discarded by White (60) because a molecular weight determination indicated a monomer rather than a dimer as required by this stucture. An oxazole (C) structure suggested by Morgan (64) was eliminated by White (60) since there appeared to be no evidence for a conjugated double bond from spectrographic data and because acetylation gave a tri-O-acetyl compound rather than a tetra-acetyl compound required by the oxazole structure.

In the course of the study of the degradation products of A-substance, it became apparent that N-acetyl glucosamine was not a particularly satisfactory absolute standard for Ehrlich's test. Thus one fraction isolated from the degradation of N-acetyl glucosamine, fraction F6B, possessed a color in Ehrlich's test equivalent to 155% N-acetyl glucosamine; it would be assumed that the reaction product would be equivalent to 109% N-acetyl glucosamine if the glucoxazoline structure was correct and complete conversion occurred. This discrepancy supports qualitative observations that the formation of the reaction product from N-acetyl glucosamine is not quantitative. For this reason it was thought desirable to compare the colors with a standard compound containing the same functional grouping as glucoxazoline and which could be prepared pure. The compound 2-methyl-Δ^2-oxazoline,  , was selected as a possible suitable

standard. This compound[1] was prepared according to the method of Wenker (63) by the distillation of N-acetyl ethanolamine. It was found, however, that this compound did not give a color test with Ehrlich's reagent. The compound, as might be expected for a stucture with one isolated double bond, showed no absorption in the ultraviolet down to 220 mu. Furthermore, this compound possesses basic properties; it is estimated from an acid-base titration that it possesses a pK_B of about 8.5. In contrast the fractions R104-F4B obtained from A-substance and F6B obtained from N-acetyl glucosamine show no basic properties in this region[2]. As a result, it would appear that the oxazoline structure (A) suggested by White is open to question[3].

Work has not progressed far enough to suggest a reason for this anomaly or to indicate an alternative structure. It should be pointed out, however, that oxazoles (64) are known to give Ehrlich's color test; the elementary analyses of the oxazole structure should be the same as the oxazoline. The ultraviolet spectra that have been determined here would suggest some conjugated structure; however, recently

---

[1] B.p. 106°-107°, picrate 157°-158° uncorr.; Lit. (63), b.p. 110°-111°, picrate 163°.

[2] On titrating between pH 8 and pH 3.5, fractions F6B and R104-F4B combine, respectively, with 8×10^{-5} and 45×10^{-5} moles of acid per gram.

[3] It should be pointed out that certain anomalies exist in White's work. Thus it is reported that 7.7% nitrogen is present in the substance giving Ehrlich's test; 2-methyl-Δ^2-glucoxazoline requires 6.9%. By comparison a similar product described above, fraction F6B, contains 6.7% nitrogen and is not pure. Furthermore, the optical rotation of the tri-acetyl derivative of the product showed a specific rotation of +36.7° while presumably the same compound prepared from penta-acetyl glucosamine had a rotation of +54°.

Cornforth and Cornforth (66) have determined the ultraviolet absorption spectrum of unsubstituted oxazole and found weak absorption below 240 mu (ε_{molar} 30 at 230 mu) but evidence for a maximum only below 200 mu.

Summary

The degradation of A-substance with alkali causes the production of reducing sugars and products giving a color with Ehrlich's p-dimethylaminobenzaldehyde reagent; at the same time A-substance is degraded in part to dialyzable low molecular weight fragments among which occurs the product giving Ehrlich's test.

The immunological properties of A-substance are retained in nondialyzable fractions. The potency of these fractions is 10-15 times greater in the hemolysis inhibition test than in the isoagglutination inhibition test.

The ultraviolet absorption spectra of the products giving Ehrlich's test isolated from A-substance and N-acetyl glucosamine are similar, maxima appearing at 230 mu and minima at about 215 mu; the latter product possesses approximately twice the color intensity of the former in Ehrlich's test and also twice the extinction at 230 mu.

The reactions leading to the compound giving Ehrlich's test from A-substance are discussed.

A few objections to the 2-methyl-Δ^2-glucoxazoline structure of the alkaline decomposition product of N-acetyl glucosamine are presented.

VIII A Laboratory Lyophil Apparatus

During the course of work on the isolation of materials in hog gastric mucin, a need arose for an efficient lyophil apparatus capable of handling liter quantities of solution. The apparatus of Campbell and Pressman (55) was inadequate since the limiting capacity of this apparatus was about 400 ml of solution. Furthermore, the apparatus could only be operated intermittently since several hours were required between lyophilizing operations for de-icing of the condenser surface. As a result a modified type of apparatus was developed which obviated these difficulties and which had the additional advantages of low cost and ease of construction. The apparatus is shown in Fig. 1.

The apparatus consists essentially of five female standard taper 34/45 joints. Four of the joints, which are arranged at right angles, serve as ports for the insertion of lyophil flasks. The flasks were constructed from male 34/45 joints and Kjeldahl flasks as described previously (55), (see item C, Fig. 1). The joints are arranged as compactly as possible in order to shorten the path of water vapor from the lyophil flask to the receiver; however apparatus with joints having arms of 2 - 3 inches still operate effectively. The fifth joint accomodates the receiver for condensing moisture; the receiver is simply another lyophil flask constructed from a 800 ml Kjeldahl flask; the receiver is replaced periodically during operation of the apparatus. The evacuation of the system was attained by means of a center tube of 12 mm Pyrex tubing attached by means of a ring seal to the apparatus. The center tube extended only as far as the joint for the receiver; the tube was extended into the receiver

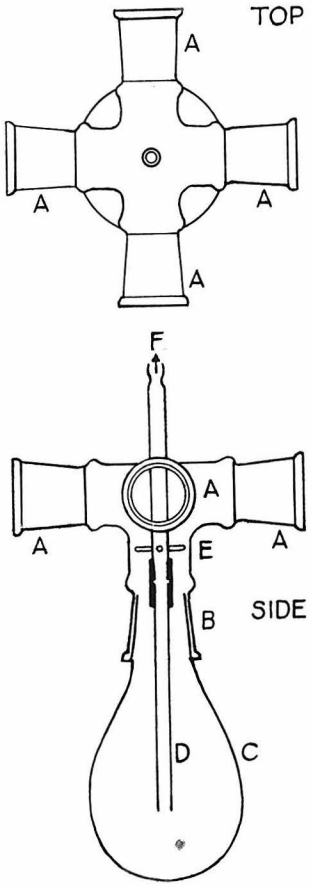


Fig. 1 . Lyophil apparatus

A Ports, ℧ 34/45, for flasks

B Port, ℧ 34/45, for receiver flask

C Receiver constructed from 800 ml Kjeldahl flask and inner ℧ 34/45 joint

D Removable tube connected to center tube with rubber tubing

E Three glass pin supports arranged symmetrically on center tube

F To vacuum

flask by means of additional glass tubing and a gum rubber connection. This arrangement relieved undue strain on the ring seal of the center tube; in addition three glass pin supports were arranged on the upper part of the center tube in order to repress futher strain on the ring seal. The glass pins were constructed so as to fit as snugly as possible against the inner wall of the joint; the direct sealing of the pins to the wall was undesirable since this would have complicated the annealing of the apparatus.

The operation of the apparatus was similar to that of Campbell and Pressman (55). The four flasks were filled with solution and frozen by turning in a bath of diethylene glycol and dry ice. The flasks were attached to the apparatus, after lubrication of joints, the receiver was put in place and the apparatus moved downward until the receiver was immersed to within a few inches of the joint in a dry ice-diethylene glycol bath. The cooling mixture was conveniently contained in a one gallon wide mouth Dewar flask. The system was then evacuated with an efficient vacuum pump (a Hyvac was satisfactory). In order to replace the receiver, air could be admitted into the system by an auxiliary manifold system. The limiting capacity of the 800 ml receiver is about 300 grams of water since this is the maximum amount of water that can be frozen in the flask safely without breakage during subsequent de-icing. De-icing was conveniently done by allowing the flask to warm slowly in air.

The efficiency of the apparatus may be judged from the data in Table 1. Approximately six hours were required to remove 200 ml of

water distributed equally between four 400 ml flasks. The time of sublimation is somewhat slower than that possible with the apparatus

Table I

Operation time (hours)	Percent total water collected in receiver
0.9	21.1
2.0	44.5
3.2	64.4
4.3	80.2
5.9	97.2

of Campbell and Pressman (55) as might be expected from the shorter path from the ice surface to condensing surface in the latter apparatus. The effectiveness of the present apparatus with large volumes of solution is evident from the results in a typical run (Table II) in which 1050 ml of a dilute solution of blood group A substance was distributed between four 800 ml flasks. The lyophilizing was essentially complete after 27 hours, the apparatus being in continuous operation during this time except for receiver changes. Care must be taken if melting of the material in the lyophil flask is to be avoided to make the final receiver exchange when sufficient ice remains in the lyophil flasks to keep the material frozen.

Table II

Operation time (hours)	Water collected (ml)
3.5	325
5.5	700
11	1015
27	1040 (complete)

This precaution may be of importance in case the material being lyophilized is denatured readily. The apparatus described has seen satisfactory service for the past two years on work on the isolation of blood group polysaccharides. The apparatus has also been used successfully in this Laboratory to dry precipitates of papain containing appreciable quantities of ethanol and water.

IX The Colorimetric Determination of Hexoses with Carbazole

The Colorimetric Determination of Hexoses with Carbazole[1]

by George Holzman, Robert V. MacAllister and Carl Niemann

The difficulty of applying classical procedures to the qualitative and quantitative determination of carbohydrate in certain biological materials has led to the development of colorimetric methods in which the carbohydrate-containing material is treated with strong mineral acids causing the formation of substances which will react with compounds such as diphenylamine, resorcinol, orcinol, indole, carbazole, etc., to give distinctively colored products. The rate at which these colored products are formed (53) and the nature of their absorption spectra are frequently sufficiently distinctive to allow their use in differentiating between the various sugars.

One of the most widely used of the above colorimetric methods is the carbazole-sulfuric acid method first described by Dische (67, 68, 49) and further developed by Gurin and Hood (33, 48) for the identification and estimation of hexoses and pentoses. The latter procedure was used by Seibert and Atno (52) for the analysis of the polysaccharides present in serum and by Knight (50) for the identification of the sugars present in influenza virus. Dische (47) has recently described a modification of the carbazole method for the analysis of uronic acids.

Although the carbazole-sulfuric acid method has been used extensively, no systematic study of the variables influencing this method appears to have been reported. Difficulties encountered in the quant-

---

[1] Paper submitted for publication to the Journal of Biological Chemistry.

itative application of the carbazole method have been commented upon (52) and indeed the significance of the carbazole reaction, or other color tests, for the qualitative identification of sugars has been questioned (51). Recently in the course of a study of the polysaccharide fractions from hog gastric mucin, we have had occasion to investigate the more important variables associated with the carbazole reaction as applied to the determination of hexoses. In the course of this study the optimum conditions for the quantitative determination of hexose were determined and certain aspects of the qualitative identification of hexoses were examined.

Experimental

Reagents. Eastman White Label carbazole was precipitated three times from a concentrated sulfuric acid solution by dilution with cold water, and the dried product recrystallized from toluene. The sugars were recrystallized from aqueous-ethanol according to conventional methods (54). Technical furfural was fractionally distilled and the fraction boiling at 80-1° and 50 mm reserved for use. In order to obtain lower blanks C.P. sulfuric acid was refluxed with potassium persulfate (20 mg/l) until a negative test for oxidizing agents was obtained with starch-iodide.

Apparatus. A Klett colorimeter, green filter No. 54, was used for all colorimetric analyses. Duplicate or triplicate analyses were always performed and the results reported are average values. A Beckman Model DU Spectrophotometer, equipped with a 1 cm cell, was used in determining spectral absorption. Intensities were measured at 10 mu intervals

except in the region of maxima and minima where the interval was reduced to 2 - 5 mu.

Effect of sulfuric acid concentration. With the amount of carbazole set at 1.5 mg, the quantity of hexose at 100 μg of glucose per ml and ten minutes for the time of heating, variation of the sulfuric acid concentration gave rise to the values given in Table I. Similar results were obtained with galactose.

Table I

Effect of Sulfuric Acid Concentration

Conc. H_2SO_4 added (wt/wt)	Klett value[1]
80	215
82	230
84	230
86	220
89	180

Effect of carbazole concentration. With the concentration of the added sulfuric acid maintained at 84 percent (wt/wt), the amount of hexose at 100 μg of glucose per ml and the time of heating at ten minutes, variation of the carbazole concentration between the limits of 1.5 mg and 4.5 mg gave rise to the values presented in Table II. Similar results were obtained with galactose.

[1] Corrected for blank on 1 ml of water

Table II

Effect of Carbazole Concentration

Carbazole present (mg)	Klett value[1]
1.5	240
2.5	316
3.0	363
3.5	402
4.5	438

Effect of time of heating. Test solutions containing 9 ml of 84 percent sulfuric acid, 3 mg of carbazole and 100 µg of glucose in 1 ml of water were heated in a boiling water bath for varying periods. It was found that 80 percent of the maximum color intensity was attained after heating for seven and one-half minutes, 99 percent after ten minutes, 100 percent after fifteen minutes and 97 percent after twenty minutes. As in other experiments galactose gave similar results.

Modified procedure[2]. A reagent was prepared by adding 10 ml of a 1.0 percent solution of carbazole in absolute ethanol to 300 ml of 84 percent sulfuric acid. Nine ml portions of this reagent were chilled in an ice bath, 1 ml of the hexose solution layered onto the reagent, the solutions thoroughly mixed and heated in a boiling water bath for

---

[1] Corrected for blank on 1 ml of water

[2] The procedure of Gurin and Hood (33) consists of heating 1.5 mg of carbazole (0.3 ml of a 0.5 percent solution in ethanol), 1 ml of hexose solution, and 9 ml of 89 percent sulfuric acid (8:1 concentrated sulfuric acid and water) for ten minutes in a boiling water bath.

fifteen minutes. After cooling in an ice bath the intensity of the color produced was determined in the Klett colorimeter. Typical results, obtained with glucose, are given in Table III.

Table III

Determination of Glucose with Modified Procedure

Glucose, μg	Klett value[1]	Average deviation
140	682	6
120	607	12
100	527	11
80	425	10
60	349	3
40	272	4
20	185	7
10	144	4
5	129	5

Discussion

It is seen from Table I that the Klett values are particularly dependent upon the acid concentration and that maximum color intensity is obtained with 82-84 percent added sulfuric acid. It was shown, by means of extinction curves, that the variations noted in Table I

[1] Average of six separate determinations

were due to differences in intensity alone. While extinction values varied widely with sulfuric acid concentration, the position of the maxima lay between 540-550 mu in every instance. However, it should be pointed out that qualitative observations of Dische (45) suggest that significant changes in respect to the position of the maxima may occur also if the sulfuric acid concentration is varied widely.

The dependence of the Klett values upon the carbazole concentration (Table II) emphasizes the necessity of precision in adding the carbazole to the reaction mixture. An error of 3 percent in the addition of 1.5 mg of carbazole (0.3 ml of a 0.5 percent solution in ethanol) would cause a corresponding variation of about four units in Klett values. It is obvious that the addition of small volumes of a carbazole solution is undesirable especially since the solvent is ordinarily absolute ethanol which is difficult to pipet accurately. While large amounts of carbazole undoubtedly increase the sensitivity of the procedure, especially since the blank values are practically constant over the range studied, the low solubility of carbazole in the diluted sulfuric acid limits the upper concentration.

In contrast to other variables the time of heating is not particularly critical provided the time is not less than ten minutes nor more than twenty minutes. A period of heating of fifteen minutes, using 84 percent sulfuric acid, appears to be a reasonable choice.

In order to simplify the procedure of Gurin and Hood (33) the carbazole was dissolved in a relatively large quantity of 84 percent sulfuric acid and aliquots of this reagent were used for analysis. This reagent simplified the procedure for routine analysis as

well as eliminated errors arising from the addition of carbazole to individual tubes. The reagent is prepared by mixing an ethanolic solution of carbazole with 84 percent sulfuric acid because solid carbazole dissolves very slowly in sulfuric acid of this concentration. Although the reagent is known to be stable for at least six hours, occasionally a green color has appeared after standing for more than twenty-four hours. It is recommended that the reagent be prepared daily as was suggested by Seibert and Atno (52).

Nitrate and ferric iron are presumed to interfere in the carbazole procedure of Gurin and Hood (33). No interference from these constituents, or from nitrite, was observed with the modified procedure. The same Klett values were obtained, within the limits of precision discussed below, in the presence or absence of 5 μg of sodium nitrate, sodium nitrite or ferric chloride when the amount of hexose present was 100 μg of glucose. Sodium nitrite imparted a faint green color, as did ferric chloride at higher concentrations, to the cold carbazole-sulfuric acid solution. However, this color generally disappears on heating.

While the modified procedure possesses the advantages of convenience and reliability, the precision would appear to be no greater than that of the original procedure when the latter is applied with extreme care. The modified procedure has a precision of 2-5 percent (Table III) in the range of 50-150 μg of glucose. The relatively low precision is still unexplained although the complexity of the reactions occurring in sulfuric acid revealed by the extinction curves discussed below suggest that experimental conditions may not be still sufficiently

reproducible.

The extinction curves for the carbazole-hexose colored products obtained by the modified procedure are shown in Fig. 1. The curves resemble qualitatively those obtained previously (50, 52). However, the color obtained with mannose does not appear to be as markedly different from that of the other hexoses as has been observed by others (50,52). It would appear that precise control of the sulfuric acid concentration is of utmost importance not only for quantitative procedures, but also in the qualitative interpretation of absorption spectra. Due to the similarity of the curves for glucose, fructose, mannose, and galactose , the low precision of the carbazole method, and because of possible interferences from other types of compounds (33), the qualitative identification of sugars by means of the modified procedure would appear to be dubious.

Relevant to the problem of spectral identification of sugars are the extinction curves in the ultraviolet that have been obtained for sugars in sulfuric acid solution in the absence of carbazole. Figure 2 shows curves obtained for 100 micrograms of sugar in 1 ml of water and 9 ml of 84 percent sulfuric acid solution after heating fifteen minutes on the water bath. The hexoses studied exhibit maxima at about 250 mu and 320 mu. Fructose, glucose, and galactose show similar curves while mannose differs in having less absorption at 320 mu than at 250 mu. The position of the maxima correspond closely to those observed for furfural under the same conditions; the apparent conversion of the hexoses to a furfural derivative would appear to be only 10-20 percent as judged from the spectra. While the mechanism

of the carbazole reaction and similar color tests are not clearly understood, presumably the formation of aldehyde intermediates are important (45,47). It is significant that N-acetylglucosamine, which does not give a color test with carbazole, also shows no specific absorption in the ultraviolet in sulfuric acid solution.

The relative heights of the maxima in the ultraviolet are not correlated with the intensity of the colors produced in the carbazole reaction, a fact which might argue for the unimportance of the compounds showing ultraviolet absorption in the subsequent color reaction. However, extinction curves of unheated sulfuric acid solutions of the hexoses (Fig. 3) suggest a complicating feature. The ultraviolet absorption spectra were found to be sensitive to time of heating and the extinction values increased and then decreased on heating successively longer periods. Glucose, fructose, mannose and N-acetylglucosamine show no appreciable absorption in the cold, while fructose is converted rapidly to an intermediate showing specific absorption in the region 250-320 mu; maximum absorption is reached after about forty minutes at 25°C. When the fructose solution is then heated, the specific absorption decreases markedly indicating other reactions leading to decomposition. Fructose also shows characteristic behavior in the carbazole reaction in that a color appears several times faster and with greater intensity than for any of the other hexoses. It is apparent that the marked difference in behavior of fructose from other sugars in cold sulfuric acid could readily be adapted to its detection under suitable conditions. Since it is known that heated acid solutions of the hexoses will not react

appreciably with carbazole in the cold (47), the carbazole reaction would appear to consist of at least two series of reactions, 1) the conversion of hexoses to intermediates showing specific ultraviolet absorption and the simultaneous decomposition of these intermediates in hot acid solution, and 2) the reaction of some or all of the products with carbazole in hot acid solution to yield a stable visible color.

Summary

The optimum conditions for the colorimetric estimation of hexoses by reaction with carbazole in hot sulfuric acid solution have been determined and a convenient procedure giving results with a precision of 2-5 percent in the range of 50-150 micrograms of glucose is described. The colors obtained with glucose, galactose, fructose, and mannose with the procedure are not sufficiently distinctive to allow their ready differentiation and identification by spectral measurements. The significance of the ultraviolet spectra of heated and unheated sulfuric acid solutions of hexoses to the problem of estimation and identification of hexoses is discussed.

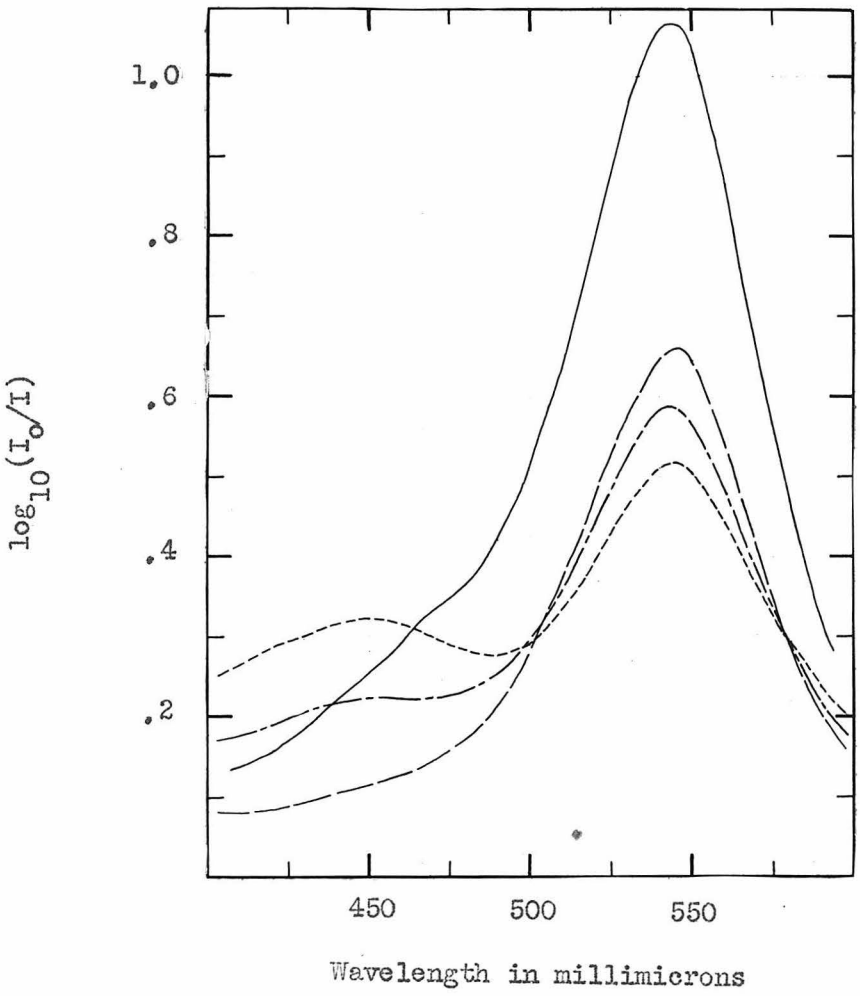


Fig. 1. Absorption curves for colors obtained with various sugars on reaction with carbazole. ———, 100 micrograms fructose; —— —, 100 micrograms glucose;__ .__, 100 micrograms galactose;- - - - -, 100 micrograms mannose.

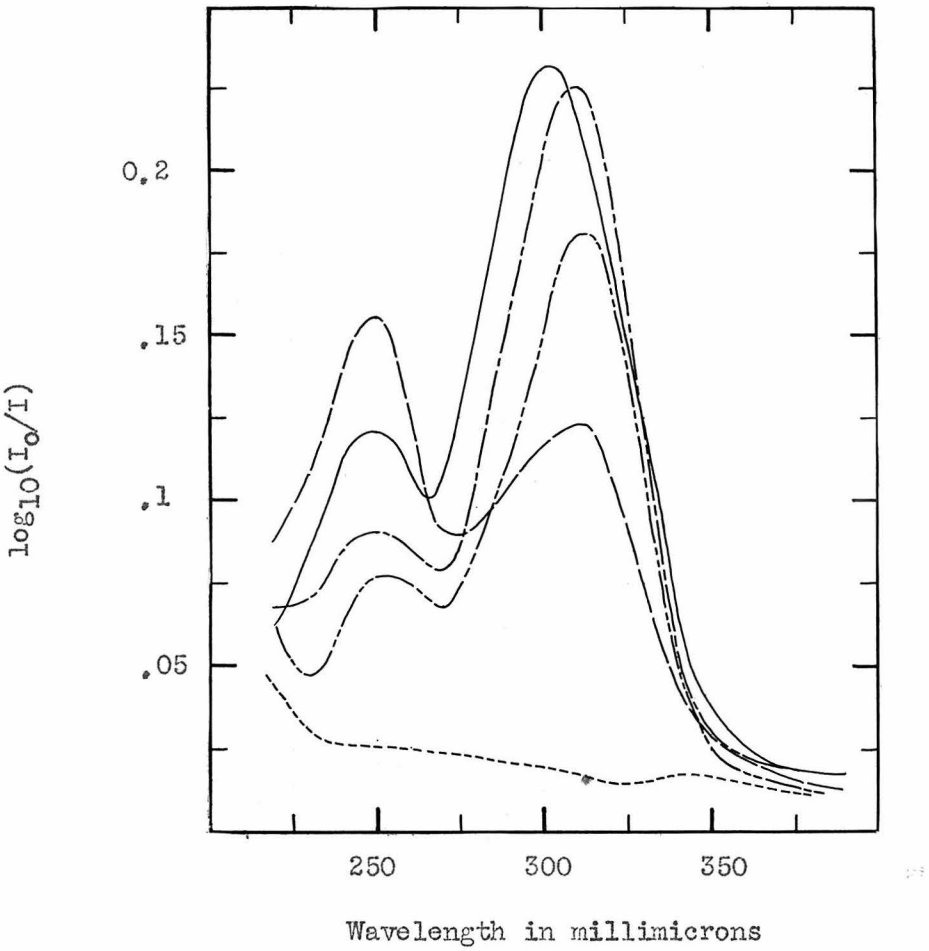


Fig. 2. Absorption curves for various sugars heated in sulfuric acid solution. ———, 100 micrograms galactose;— - —, 100 micrograms glucose;— -- —, 100 micrograms fructose; —— —, 100 micrograms mannose; - - - - -, 100 micrograms N-acetyl glucosamine.

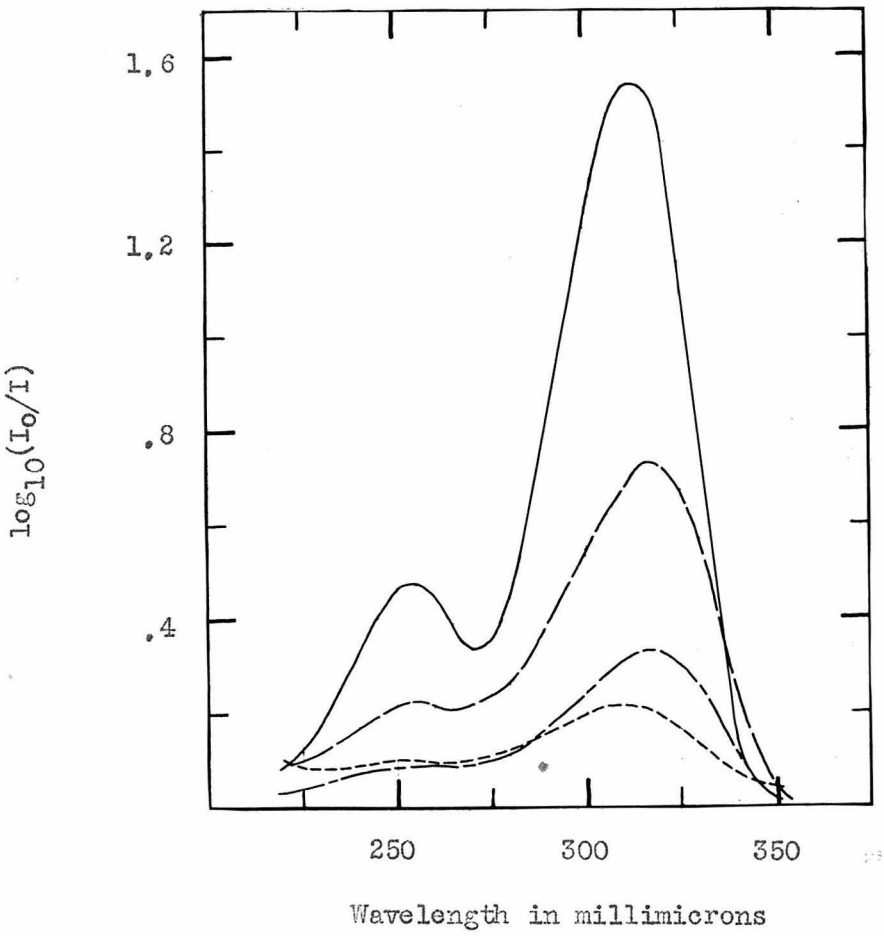


Fig. 3. Absorption curves for furfural and various sugars in sulfuric acid solution. ————, 100 micrograms furfural; — ——, 100 micrograms fructose after standing forty minutes;— - ——, 100 micrograms fructose after standing five minutes; - - - - -, 100 micrograms fructose after standing forty minutes and then heating fifteen minutes in water bath. Curves for galactose, mannose, glucose, and N-acetyl glucosamine are not show since densities are below 0.03 throughout wavelength range.

References (Parts III - IX)

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

(2) Bray, H.G., Henry, H., and Stacey, M., Biochem. J. 40, 125 (1946).

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

(4) Brown, D.H., Bennett, E.L., Holzman, G., and Niemann, C., Arch. Biochem. In press. (See Part II, this thesis).

(5) Beckman, A.O., and Cary, H.H., J. Opt. Soc. Am. 31, 682 (1941).

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

(7) Dische, Z., Proc. Soc. Exp. Biol. Med. 55, 217 (1944).

(8) Freudenberg, K., Naturwiss. 24, 522 (1936).

(9) Gróh, J., Szélyes, L., and Weltner, M., Biochem. Zeit. 290, 24 (1937).

(10) Greenstein, J.P., Advances in Protein Chemistry, V.I, p. 209, Academic Press, 1944.

(11) Goodwin, T.W., and Morton, R.A., Biochem. J. 40, 628 (1946).

(12) Gabryelski, W., and Marchlewski, L., Biochem. Zeit. 250, 385 (1932).

(13) Holiday, E.R., Biochem. J. 30, 1795 (1936).

(14) Holiday, E.R., and Ogsten, A.G., Biochem. J. 32, 1106 (1938).

(15) Holzman, G., Bennett, E.L., Brown, D.H., and Niemann, C., Arch. Biochem. 11, 415 (1946). (See Part I, this thesis).

(16) Heller, W., and Vassy, E., Phys. Rev. 63, 65 (1943).

(17) Heller, W., and Vassy, E., J. Chem. Phys. 14, 565 (1946).

(18) Kabat, E.A., Bendich, A., and Bezer, A., J. Exptl. Med. 83, 477 (1946).

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

(20) Landsteiner, K., and Chase, N.W., J. Exptl. Med. 63, 813 (1936).

(21) Loofbourrow, J.R., Rev. Mod. Phys. 12, 267 (1940).

(22) Morrow, C.A., and Sandstrom, W.M., Biochemical Laboratory Methods, p. 152, Wiley, 1935.

(23) Martin, A.J.P., and Synge, R.L.M., Advances in Protein Chemistry, Vol. II, p. 56, Academic Press, 1945.

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

(25) Meyer, K., Smyth, E.M., and Palmer, J.W., J. Biol. Chem. 119, 73 (1937).

(26) McRary, W.L., and Slattery, M.C., Arch. Biochem. 6, 151 (1945).

(27) Pardee, A., Thesis, California Institute of Technology, 1947.

(28) Stenström, W., and Reinhard, M., J. Biol. Chem. 66, 819 (1925).

(29) Witebsky, E., The New York Academy of Sciences, Conference on Blood Groups, May 18 and 19, 1945 (preprinted manuscripts).

(30) Weltner, M., Biochem. Zeit. 297, 142 (1938).

(31) Fogelson, S.J., J. Am. Med. Assn. 96, 673 (1941).

(32) Goebel, W.F., J. Exptl. Med. 68, 221 (1938).

(33) Gurin, S., and Hood, D.B., J. Biol. Chem. 131, 211 (1939).

(34) Kabat, E.A., and Bezer, A.E., J. Exptl. Med. 82, 207 (1945).

(35) Longsworth, L.G., and MacInnes, D.A., J.A.C.S. 62, 705 (1940).

(36) Morgan, W.T.J. and Elson, L.A., Biochem.J. 28, 988 (1934).

(37) Morgan, W.T.J., and Van Heyningen, R., Brit.J.Exptl. Path. 25, 5 (1944).

(38) Morgan, W.T.J., and Waddell, M.B.R., Brit. J. Exptl. Path. 26, 387 (1945).

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

(40) Niederl, J.B., and Niederl, V., Quantitative Organic Analysis, p.199, Wiley (1942).

(41) Brand, E., and Kassell, B., J. Biol. Chem. 145, 359 (1946).

(42) Schmidt, C.L.A., Chemistry of the Amino Acids and Proteins, pp. 598, 721, 932, Thomas (1944).

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

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

(45) Fogelson, S.J., U.S. Patent 1829270 (1931).

(46) Wiener, A.S., Blood Groups and Transfusion, pp. 31,280, Thomas (1943).

(47) Dische, Z., J. Biol. Chem. 167, 189 (1947).

(48) Gurin, S., and Hood, D.B., J. Biol. Chem. 139, 775 (1941).

(49) Hepburn, J.S., and Lazarchick, M., Am. J. Pharmacy, 102, 560 (1930).

(50) Knight, C.A., J. Exptl. Med. 85, 99 (1947).

(51) Meyer, K., Recent Advances in Protein Chemistry, Vol. 2, p.257, New York (1945).

(52) Seibert, F.B., and Atno, J.,J. Biol. Chem. 163, 511 (1946).

(53) Soerensen, M., and Haugaard, G., Biochem. Zeit. 360, 247 (1933).

(54) Polarimetry, Saccharimetry and the Sugars, National Bureau of Standards Circular C440 (1942).

(55) Campbell, D., and Pressman, D., Science 99, 285 (1944).

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

(57) Freudenberg, K., and Eichel, H., Ann. 510, 240 (1934).

(58) Freudenberg, K., and Eichel, H., Ann. 518, 97 (1935).

(59) Morgan, W.T.J., Biochem. J. 40, Proceedings xv (1946).

(60) White, T., J.C.S. 428 (1940).

(61) MacLeod, M., and Robison, R., Biochem. J. 23,517 (1929).

(62) Bromund, W.H., and Herbst, R.M., J.Org. Chem. 10, 267 (1945).

(63) Zuckerkandl, F., and Messiner-Klebermass, L., Biochem. Zeit. 236, 19 (1931).

(64) Morgan, W.T.J., Chem. Ind. 57, 1191 (1938).

(65) Wenker, H., J.A.C.S. 57, 1079 (1935).

(66) Cornforth, J.W., and Cornforth, R.H., J.C.S. 96 (1947).

(67) Dische, Z., Biochem. Zeit., 189, 77 (1927).

(68) Dische, Z., Mikrochemie, 8, 4 (1930).

Experiments on the Mechanism of Diurnal Acid Production in Succulents. The Carbon Dioxide Content of the Extractable Gases of Bryophyllum fedtschenkoi

The unusual metabolism of succulents which is characterized by diurnal fluctuations in the acidity of leaves and other tissues has been reviewed by a number of workers - Small (1), Richards (2), Bennet-Clark (3), Ruhland and Wolf (4), Rabinowitch (5) and recently by W. Bonner (Thesis, C.I.T., 1946). In most of the succulents organic acids, principally malic, citric, and isocitric acids accumulate during the night and decrease markedly during the day. Some evidence exists that the appearance of acids is associated with respiration (5), e.g., it is known that organic acids accumulate at the cost of sugars. Similarly the disappearance of acids is associated presumably with the role of the acids as photosynthetic intermediates in as much as in light and in the absence of carbon dioxide carbohydrates accumulate with a concomitant loss of organic acids (5). However deacidification in the dark appears possible under certain conditions.

The experiments described in this section were initiated at the suggestion of Professor James Bonner as a test of a new hypothesis (W. Bonner) concerning the formation of organic acids in succulents. Experiments of W. Bonner indicated that the production of organic acids in detached leaves of Bryophyllum fedtschenkoi was dependent on the carbon dioxide concentration in the surrounding atmosphere. Approximate maximal production of organic acids was attained at 0.1% carbon dioxide, a value close to the actual carbon dioxide content of the atmosphere. On the basis of these and supplementary observations it was postulated that the carbon dioxide content of the internal atmosphere of leaves controlled the nightly production of organic acids. It was assumed that the carbon dioxide content would be lower in the

fleshy leaves of the succulents during the day, thus accounting for low organic acid content, than at night due to the utilization of carbon dioxide for photosynthesis. Accordingly a test of this hypothesis was made by assessing the carbon dioxide content of the internal atmosphere of the leaves of Bryophyllum fedtschenkoi at various times during the night and day.

Experimental

Apparatus. The gas extraction apparatus is shown in Fig. 1 . The carbon dioxide analyses were made with a Van Slyke manometric apparatus.

Description of plants. For most of the experiments the leaves studied consisted of the fairly mature forms from Bryophyllum fedtschenkoi growing in individual pots in the open. The plants had been used previously in studies on the organic acids of leaves (W. Bonner, Thesis, C.I.T. 1946). In experiment 7, however, the plants were acclimated in the dark for 10 days in a greenhouse at 21-22°C before leaf sampling. For all experiments except No. 7 the leaves were removed from the same lot of about three dozen plants. For each experiment about 50 leaves were taken from about 8-10 different plants, each leaf weighing between 0.5 and 1 gram. The leaves evolved gas amounting to between 6-7% of their total volume under the conditions of gas extraction. The plants used in experiment 7 differed in that these plants bore leaves of considerably larger size. Each leaf weighed between 2-2.5 g; about 12-13 leaves were used and it was found that gas amounting to 10-12% of the volume of the leaves was

extractable.

Sampling of leaves. The leaves were removed from plants by pinching off at the base of the petiole. The leaves were packed into the receiver of the gas extraction apparatus preparatory to returning to the laboratory for the gas analysis. The ambient air temperature in the open or in the green house during sampling was recorded with the aid of an ordinary mercury thermometer. The sampling was performed as rapidly as possible; in general about 10 minutes were required for sampling the leaves and 30-45 minutes additional time before the gas extraction in the laboratory was completed. In Experiment 7, in which the effect of the time of insolation was studied, the sampling usually required about 5 minutes and the gas extraction was completed about 20-25 minutes later. The leaves were weighed in the receiver immediately after sampling.

Apparent volume change of leaves after gas sampling. The receiver containing the leaves was capped and weighed. Mercury was then admitted into the receiver through one stopcock until it rose in the bore of the other stopcock. The stopcocks were closed, the receiver shaken, and a small bubble of air occluded on the leaves or glass was ejected. This process was repeated 3-4 times until no appreciable bubble of air was detectable. The receiver containing mercury and leaves was then weighed. Finally the receiver was attached to the gas extraction apparatus and after gas extraction the receiver, filled with mercury, was weighed again. From the volume of the receiver assembly and the weight of mercury displaced by leaves it was possible to calculate the apparent volume of leaves before and after gas extraction. The

apparent volume of leaves always showed a decrease of 1-3% after gas extraction. Typical results are shown in Table II. The density of leaves was 0.98-.99.

Extraction of gas. The gas was extracted from the leaves with a modified Toepler pump designed by Mr. Victor Schocken (see Fig. 1). The sequence of operations was as follows: the pressure was reduced in the mercury reservoir until the upper bulb of the Toepler pump was free of mercury and connected directly to the receiver system. Gas evolved rapidly from the leaves in the receiver and distributed between the upper bulb of pump and the connecting tubing; the leaves in the receiver were always covered with mercury. The pressure in the mercury reservoir was increased to atmospheric pressure and the entrapped air in the upper bulb was forced into the gas sampling pipet. After measurement of the height of the mercury meniscus in the gas sampling pipet above the meniscus in the mercury reservoir the system was ready for the next cycle in the gas extraction. Each extraction required 1-2 minutes. One gas extraction usually collected 95% of the gas while two extractions collected essentially 100%. Further extraction produced negligible quantities of gas. After the gas extractions (usually four were made) the sampling pipet was removed and weighed.

The volume of gas collected (Method I) was estimated from the calibrated volume of the sampling pipet and the volume displaced by the mercury in the partially filled pipet. The pressure in the gas space was corrected for the unknown amount of water vapor extracted from the leaves by assuming saturation of the air space. The maximum increase in the carbon dioxide content of the air resulting from this

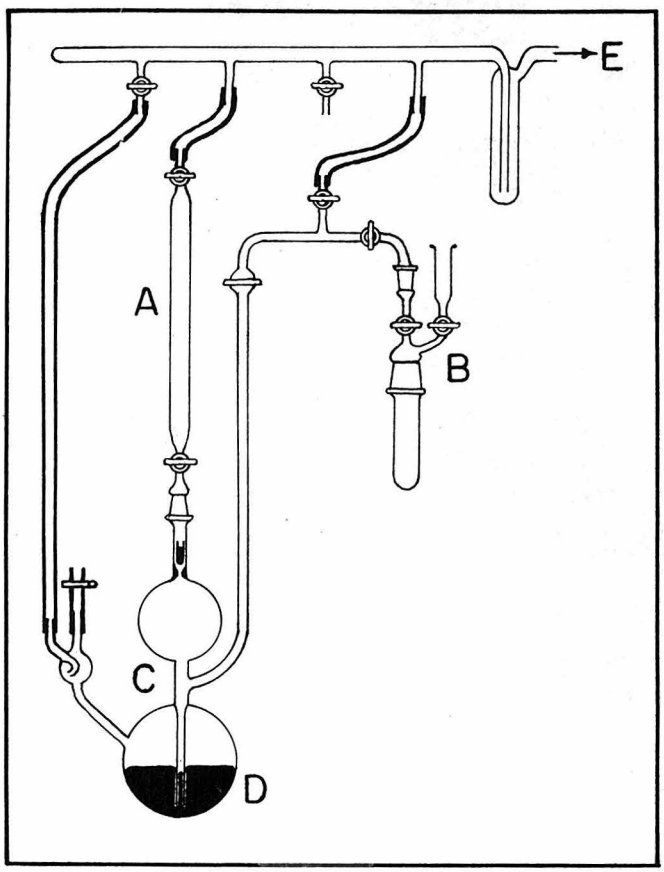


Fig. 1 . Gas extraction apparatus

A Gas sampling pipet

B Receiver containing leaves

C Toepler pump

D Mercury reservoir

E To vacuum pump

assumption is about 10%. Results are shown in Table II. In a few instances the total volume of gas collected was estimated also in a less precise way in the following manner (Method II). The gas sampling pipet was connected to an evacuated Van Slyke gas analysis apparatus through the sidearm of the Van Slyke pipet by means of mercury-filled rubber connections. The pressure of the system (10 ml of air-free water was added to the Van Slyke pipet) was then measured at the 10, 2, and 0.5 ml marks of the Van Slyke pipet. From the pressures at these volumes, corrected for zero readings (system not containing air) it was possible to calculate the volume of gas collected using Boyle's law. Results were obtained by solving the equation,

$$P_1(V_1 + V_o) = P_2(V_2 + V_o)$$

$$\text{or } V_o = \frac{P_2V_2 - P_1V_1}{P_1 - P_2},$$

where V_o is the volume of gas collected at atmospheric pressure, V_1 and V_2 are the volumes of the Van Slyke pipet at two different calibration marks, and P_1 and P_2 the pressures when the system was at volumes $V_1 + V_o$ and $V_2 + V_o$ respectively. Two other values of V_o are obtained when the pressure is measured at a third calibration mark by pairing off the various values of P and V. The method is inaccurate since it is dependent on the measurement of differences which may be small. However the method is useful since it provides an independent method of checking the carbon dioxide concentration in the extracted gas by the measurement of V_o before and after the addition of alkali. The results in Table II indicate that the decrease in volume after the addition of alkali is in agreement within experimental error with that estimated from the carbon dioxide content of the gas

by the precise method below.

Carbon dioxide content of the extractable gases. The gas sampling pipet was attached to the sidearm of the Van Slyke pipet by means of murcury-filled rubber connections. The usual procedure for the absorption, evolution, and measurement of carbon dioxide was performed. The carbon dioxide was absorbed in 10 ml of air-free water and 2 ml of 0.016 M NaOH. The carbon dioxide was liberated with 0.2 ml of 2 M lactic acid and the pressure of carbon dioxide measured at the 0.5 ml mark. In a few analyses only 1 ml of alkali was used. A blank analysis was carried out under the same conditions.

As a check on the apparatus a carbon dioxide analysis was carried out in 10 ml of laboratory air; less than 0.1% by volume of carbon dioxide was found. Due to difficulties in transferring the air from the sampling pipet to the Van Slyke pipet and to the inaccuracies of measuring pressures at small volumes in the Van Slyke apparatus, the deviations in the carbon dioxide content of 2-4 ml air samples may be as large as $\pm$ 0.2-0.3 volume percent carbon dioxide. The limiting concentration of carbon dioxide which is detectable is about 0.2 volume percent on similar aliquots of air.

Approximate relative buffer capacity of leaves. After the gas extraction of leaves, the mercury was removed from the receiver containing the leaves. Ten grams of leaves were taken and crushed in a mortar; the pH of the crushed leaves was measured with a Beckman pH meter. Then 5 ml of water was added to the leaves and the suspension titrated with 1.00 N NaOH with the aid of the Beckman meter. The relative buffer capacity was estimated from the milliequivalents of NaOH pergram

NaOH per gram of leaves required to bring the pH of the crushed leaves to 7. Results are shown in Table I.

Results and Discussion

In Table I are summarized the diurnal variations in acid production of Bryophyllum fedtschenkoi in day and night experiments and in Table II are shown corresponding results obtained for the apparent carbon dioxide content of the extractable gases of the same leaves. The results in Table I confirm previous observations as to a marked diurnal fluctuation in the acidity of leaves. The pH of the expressed sap varied from 4.2 at night to 5.4 during the day; a similar fluctuation in the relative buffer capacity of leaves occurred although these results are not to be considered due to organic acids solely since phosphate, for example, would contribute buffer capacity under these conditions.

From the results in Table II it is seen that the apparent carbon dioxide content of the gases of the leaves during night and day is precisely opposite to that expected if the hypothesis being tested was valid. During the night the leaves had a carbon dioxide content of 2-4% while during the day the carbon dioxide content was 7-16%[1]. However these results cannot be considered

---

1 These values are the analytical concentrations of carbon dioxide in the total gas extracted from 40-50 leaves. The values are minimal since the total gas extractable is increased somewhat by gas apparently absorbed on glass or leaves or by small air bubbles in the receiver containing leaves. This extraneous gas was detected by measuring the apparent volume of leaves before and after extraction of gas; the volume changes are shown in column 5 of Table II. The

Table I

Diurnal Variation in Acid Production of

Leaves of Bryophyllum fedtschenkoi

Expt. No.	Time of sampling	pH of crushed leaves	Approximate relative buffer capacity of leaves (milli-equivalents of NaOH/gram leaves required to obtain pH 7)
1	Day 25 July 1946 11:40 AM	-	-
2	Night 17 August 1946 12:20 AM	4.26	0.110
3	Day 22 August 1946 2:00 PM	5.42	.026
4	Night 22 August 1946 11:10 PM	4.54	.076
5	Day 31 August 1946 2:00 PM	4.99	.046
6	Night 31 August 1946 10:05 PM	4.60	.075

Table II

Diurnal Variation in the Carbon Dioxide Content of the Extractable Gases of Leaves of Bryophyllum fedtschenkoi

Expt.	Time of sampling	Ambient air temp. during leaf harvest (°C)	Wt. of leaves (g)	Volume change of leaves on sampling (ml)	Volume* of gas collected				Volume* of CO_2 found in gas (ml)	Ml of gas per gram of leaves (Method I)	Ml of CO_2 per gram of leaves	Apparent volume percent CO_2 in collected gas
					Method I (ml)	Method II						
						Volume before CO_2 absorption (ml)	Volume after CO_2 absorption (ml)	Volume change on CO_2 absorption (ml)				
1	Day 25 July 1946 11:40 AM	-	51.80	-	3.9	-	-	-	0.290	0.076	0.0056	7.4
2	Night 17 August 1946 12:20 AM	16	46.68	-	2.8	-	-	-	.089	.060	.0019	3.2
3	Day 22 August 1946 2:00 PM	35	41.09	-1.1	2.9	-	-	-	.248	.071	.0060	8.6
4	Night 22 August 1946 11:10 PM	20	31.50	- .6	2.1	2.0	1.9	-0.1	.056	.068	.0018	3.9
5	Day 31 August 1946 2:00 PM	34	38.00	- .5	2.7	2.7	2.4	- .3	.435	.072	.0144	16.0
6	Night 31 August 1946 10:05 PM	18	36.65	- .5	2.3	2.0	2.0	.0	.054	.062	.0015	2.4

* Volumes calculated at 25°C and one atmosphere and corrected for presence of water vapor.

to be a critical test of the hypothesis regarding carbon dioxide in the atmosphere and organic acid production in leaves since the values found for the carbon dioxide content in the extractable gases during night and day are 60 to 500 times greater than the carbon dioxide content of the atmosphere (0.03%). Since it is unlikely that the carbon dioxide content of the intercellular space is continually higher than the atmospheric content - this would require some mechanism for concentrating carbon dioxide against a concentration gradient - it must be concluded that the carbon dioxide measured in the extractable gases was not actually existent as a gas in the intercellular space. Therefore the experiments reported here cannot test directly any relation between the internal atmosphere of leaves and acid production. However the ready extractability and the large amount of carbon dioxide present and the significant difference between results during night and day must be considered as important factors in any attempt to assess the significance of external carbon dioxide concentration.

Similar observations as to the high apparent carbon dioxide

---

1 (concluded from previous page) magnitude of the change is variable and thus cannot be considered due only to the compression and consequent diminution in volume of leaves after removing the intercellular gases by extraction. In no instance would a correction for the volume change so alter the carbon dioxide concentrations of the gases such that the carbon dioxide content was higher at night than during the day.

The total volume of gases extracted, about 10% of volume of leaves, appears a reasonable value which might be expected for the intercellular volume in as much as Turrell (12) has found that the related species of succulent, Bryophyllum calycinum, has 17% volume of intercellular space based on microscopic measurements of leaf sections.

content of leaves of non-succulent plants have been reported previously. Shafer (6) observed values of 1-3% carbon dioxide in gases from leaves of broad bean (Vicia faba), beet, nasturtium, willow and rose. However only in the case of Vicia faba was a small but significant difference between the carbon dioxide content in day and night experiments demonstrable. It should be noted that the results reported here for the carbon dioxide contents of gases from Bryophyllum fedtschenkoi during the day are 4-8 times greater than at night, a fluctuation which is much greater than the maximum of about one-half greater carbon dioxide content during the day than at night found by Shafer (6) for Vicia faba; it would be of interest to ascertain whether great fluctuations of carbon dioxide content are characteristic for plants showing succulent-type metabolism. Laing (7) observed high concentrations of carbon dioxide in gases from the rhizomes and petioles of water plants although these results are somewhat complicated by anaerobic respiration. Earlier observations on the internal atmosphere of leaves are apparently unreliable (6).

Since it seems unreasonable that the carbon dioxide in the extractable gases existed as such in the intercellular space it must be assumed that the carbon dioxide was bound physically or chemically. Thus Smith (8,9) was able to account for the carbon dioxide absorption of a number of plants by means of 1) physical solution in the water of the sap, 2) chemical reaction with buffer substances, e.g., $HPO_4^{=}$, and 3) by the dissolution of insoluble alkaline earth carbonates. However none of these factors can be of importance in explaining

the high carbon dioxide content of the extractable gases of the succulent studied. For example, the physical solution of carbon dioxide in a volume of water equivalent to that of the leaves could account for only 2-10% of the carbon dioxide observed. The low pH of the succulents obviates any combination with the known buffering substances to form bicarbonate or carbonate.

Shafer (6) postulated a light-activated mechanism by which carbon dioxide was bound in as much as it was observed that the carbon dioxide in the extractable gases of Vicia faba decreased significantly (about 30%) after the plants were placed in the dark for thirty minutes. However, these results offer no explanation for the residual high carbon dioxide content of the gases in the leaves in the dark (1-1.5%). It is questionable whether a light-activated process is of importance for the leaves studied. Table III shows that when leaves placed in the dark were exposed to light there was no evidence of a significant increase in the carbon dioxide content of the extractable gases. The carbon dioxide content of the evolved gases was essentially the same after 0, 10, and 30 minutes insolation, 17-19%[1]; the leaves also showed no marked change in the amount of acid present. The pH of the crushed leaves after 0, 10, and 30 minutes of insolation were, respectively, 4.3, 4.3, and 4.2; the corresponding relative buffer capacities (see Table I for units) were 0.15, 0.13, and 0.14.

---

1 The carbon dioxide content of the gases from leaves in the dark is somewhat larger than that found previously (Table I). It is not known to what extent the conditioning of plants to dark influenced this result.

Table III

Effect of Insolation on the Carbon Dioxide Content of Extractable Gases of Leaves of Mature Bryophyllum fedtschenkoi Grown in Dark for 10 Days (21-22°C)

Experiment 7

Sample	Time of insolation (minutes)	Ambient air temp. during leaf harvest (°C)	Wt. of leaves (g)	Volume change of leaves on sampling (ml)	Volume* of gas collected Method I (ml)	Volume* of CO_2 found in gas (ml)	Ml of gas per gram of leaves	Ml of CO_2 per gram of leaves	Apparent volume percent CO_2 in collected gas
1	0	22	28.0	-0.3	3.1	0.604	0.111	0.0216	19.3
2	5-10	22	30.0	-1.2	3.8	.647	.127	.0216	16.9
3	32-37	23-24	36.6	- .8	3.6	.700	.099	.0191	19.3

* Volumes calculated at 25°C and one atmosphere and corrected for the presence of water vapor.

It is evident that sufficient data are not yet available to explain the origin of the carbon dioxide in the extractable gases. A possible source of the carbon dioxide is from the carbon dioxide complex formed in the first step of the photosynthetic reaction (5). The fixation of radioactive carbon dioxide in the dark has been demonstrated for barley, sunflower, and wheat by Ruben, Hassid, and Kamen (11) and for Chlorella by Ruben, Kamen, and Hassid (10). In the latter experiments it was shown that the carbon dioxide complex formed is presumably dissociable and could thus afford carbon dioxide on evacuation.

Summary

The carbon dioxide contents of the extractable gases of leaves of Bryophyllum fedtschenkoi were determined on plants during night and day; the gases contained 2-4% carbon dioxide during the night and 7-16% during the day. It was not possible to test a hypothesis concerning the control of acid production in succulent plants by the carbon dioxide concentration of the internal atmosphere since it is shown that the latter quantity was not in fact measured by the technique used.

The significance and possible origin of the high carbon dioxide content of the extractable gases is discussed.

References

(1) Small, J., Hydrogen-ion Concentration in Plant Cells and Tissues, Protoplasma-Monographien, V.2, 1929.

(2)[1] Richards, H.M., Carnegie Inst. Public. 209 (1915).

(3a)[1] Bennet-Clark, T.A., New Phytologist 32 37, 128, 197 (1933).

(3b) Bennet-Clark, T.A., Ann. Rev. Biochem. 6 579 (1937).

(4) Ruhland, W., and Wolf, J., Ann. Rev. Biochem. 3 513 (1934).

(5) Rabinowitch, E.I., Photosynthesis and Related Processes V. 1 (1945).

(6) Shafer, J., Plant Physiol. 13 141 (1938).

(7) Laing, H.E., Am. J. Bot. 27 861 (1940).

(8) Smith, J.H.C., Plant Physiol. 15 183-224 (1940).

(9) Smith, J.H.C., and Cowie, D.B., Plant Physiol. 16 257 (1941).

(10) Ruben, S., Kamen, M.D., and Hassid, W.Z., J.A.C.S. 62 3443 (1940).

(11) Ruben, S., Hassid, W.Z., and Kamen, M.D., J.A.C.S. 61 661 (1939).

(12) Turrell, F.M., Am. J. Bot. 23 255 (1936).

---

1 References not available for review.

Acknowledgements

I am grateful to Professor Carl Niemann for his direction in this research and for helpful discussions during the course of the work. I wish to acknowledge the pleasant collaboration of Mr. David H. Brown and Mr. Edward L. Bennett on a considerable part of the research and their generous assistance at all times. I am indebted to Professor James Bonner for suggesting the research problem in plant physiology and for advice during the investigation. The assistance of my wife in the preparation of this thesis is appreciated.

Propositions

I I propose an extension of a method for determining the vapor pressure of liquids on a milligram scale to the determination of the molecular weight of non-volatile solutes.

C.W. Gould, G. Holzman, and C. Niemann, Anal. Chem. 19 204 (1947)

II The experimental approach to the definition of homospecific proteolytic enzymes by Bergmann has depended on the measurement of the proteolytic coefficient, i.e., the quotient of a) the unimolecular hydrolysis constant for the substrate and b) the enzyme concentration. It is proposed that the concept of homospecificity might be further examined by a consideration of the combining constants of enzyme and substrate.

M. Bergmann, Advances in Enzymology, Vol. II, Academic Press

III The structure postulated by White* for the alkaline degradation product of N-acetylglucosamine, 2-methyl-4',5'-glucopyrano-Δ^2-oxazoline, is open to criticism. The oxazoline structure could be tested critically and the possibility of an oxazole structure could be decided by means of a periodate oxidation.

T. White, J. Chem. Soc. 428 (1940)

Thesis, p. 175

IV Direct tests of homogeneity (ultracentrifuge and electrophoresis) and relatively inaccurate immunological tests are not reliable guides to the isolation of homogeneous blood group A substance from the complex mixture of materials arising from hog gastric mucosa. It is

proposed that the correlation of immunological data with tests for chemical and physical properties of all the fractions obtained in a quantitative fractionation provides a method for progressing to a preparation of constant composition.

Thesis, p.65

V It is proposed that the detection of fucose and mannose in blood group A-substance from pepsin and the assignment of various sugars to end group positions as reported by Bray, Henry and Stacey are uncertain due to the experimental technique used.

H.G. Bray, H.Henry, and M. Stacey, Biochem. J. 40 125 (1946)

VI a) The possibility of the occurrence of immunologically active components in A-substance preparations differing as to degradation, size, or composition makes the quantitative precipitin test of Kabat, Bendich and Bezer unreliable as a test for the absolute purity of A-substance preparations.

E.A. Kabat and A.E. Bezer, J. Exptl. Med. 82 207 (1945)

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

Thesis, pp. 138, 149

b) The isoagglutination inhibition test for the estimation of A-substance is a valuable characterization test due to its sensitivity to degradation in A-substance but suffers the disadvantage of low precision ($\pm$ 50%) . It is proposed that the precision of the test could be considerably improved by the use of fractionated anti-A serum and with the aid of ultraviolet spectrophotometry for detecting changes in composition of test systems.

Thesis, p.5

VII The rate of penetration of auxin (3-indole acetic acid) through the cell membrane of Nitella has been reported by Albaum, Kaiser, and Nestler to be controlled by the external concentration of the undissociated species of acid. I propose that the data presented by the authors would suggest that penetration of undissociated species of acid merely represents a superimposition on some other mechanism of accumulation of auxin.

H. G. Albaum, S. Kaiser, and H.A. Nestler, Am. J. Bot. 24 513 (1937)

VIII a) It is proposed that the origin of the high apparent carbon dioxide content of the internal atmosphere of leaves of Bryophyllum fedtschenkoi cannot be accounted for by usual processes of dissolving of gas in tissue solution or by reaction of carbon dioxide with buffer substances. An explanation open to experimental testing is that the carbon dioxide represents a part of the dissociable complex formed in the dark reaction of photosynthesis.

S. Ruben, M.D. Kamen, W.Z. Hassid, J.A.C.S. 62 3443 (1940)

Thesis, p. 204

b) The succulent, Bryophyllum fedtschenkoi, is a better test plant for examining the significance of differences in night and day carbon dioxide contents of the internal atmosphere of leaves than any of plants described by Shafer.

J. Shafer, Plant Physiol. 13 141 (1938)

Thesis, p.204

IX I propose a) a convenient apparatus for the electrodialysis of large quantities of blood group A substance solution and b)

an economical lyophil apparatus, capable of continuous operation, for handling liter quantities of aqueous solution.

Thesis, pp. 68, 179

X In the conventional systems of qualitative organic analysis (Kamm, Shriner and Fuson) emphasis on the classification of compounds by physical property measurements, as,for example, solubility, melting point, and boiling point, often leads in the laboratory to the use of functional group analysis simply for the confirmation of alternative structures in lists of possible unknown compounds. It is proposed that the use of functional group analysis on an equivalent basis in the classification of compounds is of considerable practical and pedagogical value. A necessary prerequisite to applying systematic functional group analysis would be the determination of the specificity, reliability, and sensitivity ofmany of the tests in the usual texts; it would be desirable to consider also those tests which are well known in special fields, such as alkaloids, sugars, proteins, etc. The adaptation of tests to reactions in capillary tubes or other micro equipment provides a method for maintaining economy of materials and time for such a program.