worked out it will be found to have more of the structure of the galactan in common than merely the singly linked terminal galactose residues.

Although the tamarind seed polysaccharide precipitates less of the Type XIV antibody than does the lung galactan, its reaction with the antiserum resembles the homologous type-specific reaction much more closely. The quantities necessary to reach the maximum are small, and an excess of polysaccharide causes inhibition of precipitation. In view of the present lack of knowledge of the exact structure of the galactose units in S XIV, the significance of these observations is difficult to assess.

NEW YORK 32, N. Y.
COLUMBUS 10, OHIO

[CONTRIBUTION FROM TULANE UNIVERSITY SCHOOL OF MEDICINE]

A New Procedure for the Isolation of Blood Group A Substance from Human Ovarian Cyst-Fluids[1]

BY HAROLD BAER AND INGEBORG NAYLOR

RECEIVED NOVEMBER 16, 1954

Material with blood group A activity has been isolated from two pseudomucinous ovarian cyst fluids by the process of electroconvection. The substances so isolated contain more nitrogen, probably as protein, and exhibit greater activity in the test for inhibition of hemagglutination than do substances prepared by a phenol-extraction method. In contrast to the phenol-extraction procedure that frequently yields at least two fractions with blood group A activity, the present method leads to the isolation of a single substance. The substance, in addition, has been found to be antigenic *per se* for the rabbit while other procedures have yielded material non-antigenic for the rabbit unless coupled to a protein.

Although several methods have been described for the isolation of substances having blood group specific A, B and O activity from human and animal sources, the procedure most frequently employed in recent years has been the phenol-extraction method originally devised by Morgan and King.[2,3] One feature that the various methods possess in common is the inclusion of procedures for the removal of protein, the resulting products being largely polysaccharide but containing some amino acids, probably as peptide. While seeking a simpler and somewhat less drastic isolation procedure than those previously described for the isolation of blood group substances from pseudomucinous ovarian cyst fluids, we found that paper electrophoresis could separate the active material from several inactive components. To obtain larger quantities of active substance, pseudomucinous ovarian cyst fluids from Group A secretors were subjected to fractionation by electroconvection; by this means a fraction was obtained that possessed the serological activity expected for blood group A substance. This fraction showed a higher nitrogen content than that of materials isolated by other procedures and gave reactions suggesting that it might be a polysaccharide-protein complex. This fraction had greater activity, as determined by the test for inhibition of hemagglutination, than substances isolated by the phenol-extraction method. In addition this product was antigenic *per se* for the rabbit, in striking contrast to previously studied material with blood group A activity that required coupling to a protein of *Sh. shigae*.[4-6]

Materials and Methods

Two human pseudomucinous ovarian cyst fluids were obtained from patients in the Sara Mayo Hospital of New Orleans through the kind coöperation of Dr. William Harris, Jr. Both cysts showed blood group A activity as determined by the test for inhibition of hemagglutination. Before being used for the experiments herein described, the fluid from cyst 9 had been stored for approximately a year, and that from cyst 13 for a month. During storage both materials were kept at 4–6° with added chloroform as preservative. The clear viscous liquid obtained by centrifugation of the cyst fluids was dialyzed against phosphate buffer *p*H 6.8–6.9 (prepared by dissolving 17.6 g. of KH_2PO_4 and 28.6 g. of Na_2HPO_4 in water to a volume of 5 liters) and was then placed in the electroconvection[7] apparatus, the buffer employed being the same as that against which it was dialyzed. A current of approximately 0.4 amp. and 25 volts was then passed through the apparatus for periods of time varying from about 20 to 100 hours, the temperature being maintained at 4–6°. The top and bottom fractions were removed and analyzed separately. It was possible to ascertain by visual observation that some fractionation had occurred since the bottom portion was brown while the top portion was a clear, colorless, slightly opalescent fluid.

Portions of the original cyst fluid and the top and bottom fractions were then subjected to analysis by means of paper electrophoresis.[8] The solutions were placed in the form of a band, 0.1 ml. of solution per inch of paper, on strips of Whatman No. 3 filter paper that had been previously dipped into buffer and blotted between paper towels to remove excess fluid. After the application of 250 volts for about 8 hours the strips were removed, dried in an oven at 110–120° for 20–30 minutes and treated for 5 minutes with a staining solution prepared by dissolving 40 g. of mercuric chloride and 0.5 g. of brom phenol blue in 400 ml. of 95% alcohol. The strips were then washed in running water until blue bands could be observed against a background essentially free of blue color. To separate sufficient material to permit analysis of the various zones, papers 6 in. wide were employed, the material to be separated and analyzed being pipetted onto a 5-in. section while a smaller separate portion was placed on the edge of the strip. After completion of the separation the edge strip was cut from the large sheet, dried and stained as described above; the main strip of paper was then cut into sections 10–15 mm. wide using the edge strip as a guide. Each section was extracted by being

(1) This investigation was supported by a research grant (RG 2964) from the National Microbiological Institute, National Institutes of Health, Department of Health, Education and Welfare.

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

(3) E. A. Kabat and A. E. Bezer, *J. Exp. Med.*, **82**, 207 (1945).

(4) W. T. J. Morgan, *Brit. J. Exp. Path.*, **24**, 41 (1943).

(5) W. T. J. Morgan and W. M. Watkins, *ibid.*, **25**, 221 (1944).

(6) S. J. Rainsford and W. T. J. Morgan, *Lancet*, **1**, 154 (1945).

(7) Purchased from the E. C. Apparatus Co., 23 Haven Ave., New York 32, N. Y.

(8) F. V. Flynn and P. de Mayo, *Lancet*, **2**, 235 (1951).

placed into a test-tube containing 10 ml. of 0.85% sodium chloride solution and permitted to soak for 1–2 days at refrigerator temperature. After partly disintegrating the papers with a glass rod the tubes were centrifuged and the supernate removed. In addition, a portion of the fluid from cyst 9 was subjected to trichloroacetic acid precipitation and phenol extraction[2,9,10] so that blood group substances isolated by this procedure could be compared with that obtained by electroconvection.

The analytical values for total nitrogen, fucose and total reducing sugar after hydrolysis were obtained by the methods previously employed.[10] Blood group activity was determined by inhibition of hemagglutination,[10] the anti-A serum employed being a human typing serum.[11] For use, the antiserum was diluted in such a manner that the serum-red cell control gave only a 3+ reaction. A 3+ agglutination is one in which the cells are contained in several large clumps in contrast to a 4+ reaction in which one large clump is formed.

Results

To determine whether migration in an electrical field would aid in the separation of blood group active substances, some of the whole cyst fluid was placed on paper strips at *p*H 2.5 using citrate buffer, *p*H 4.9 using acetate buffer, and *p*H 6.9 using phosphate buffer and 8.3 using veronal buffer, then electrophoresis was allowed to progress for about 8 hours. After staining, examination of the papers showed that at *p*H 4.9, 6.9 and 8.3 several distinct bands were observed whereas at *p*H 2.5 a somewhat diffuse blue staining band was present near the starting line while a large strip of paper between the starting line and the anode was stained blue but no distinct bands were obtained. Using stained edge strips as guides, papers prepared at each *p*H were cut into sections and after elution in saline and adjustment of the *p*H to approximate neutrality where necessary, were analyzed for activity by inhibition of hemagglutination. In each case the active fraction was found very nearly at the starting point and associated with a blue staining area. At *p*H 4.9 the bands staining deeply blue but devoid of activity also showed very little migration whereas at *p*H 6.9 and 8.3 there was marked separation. In appearance these papers looked similar to papers obtained by electrophoresis of the bottom fraction removed from the electroconvection apparatus, Figs. 1 and 3. Since, of the range of acidities tested, *p*H 6.9 seemed to provide a good separation, 50–100 ml. portions of the cyst fluids were dialyzed against phosphate buffer at *p*H 6.9 and after clarification by centrifugation, were subjected to fractionation in the electroconvection apparatus. Following separation of the top and bottom fractions, a portion of each was placed on paper 6 in. wide as previously described and following electrophoresis, the paper containing the top fraction was cut into 6 strips while the paper containing the bottom fraction was cut into 8. Each fraction was analyzed with the results shown in Figs. 1–4. The diagrams below the graphs provide a graphical representation of the actual papers and indicate the manner in which papers were cut into strips.

The blood group activity was localized largely in one strip in both the top and bottom fractions. The

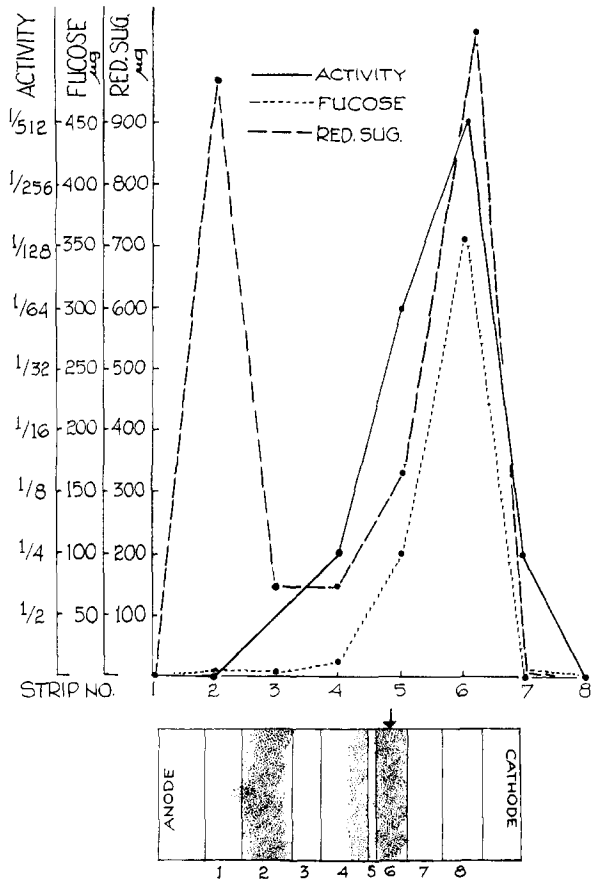

Fig. 1.—Analysis of segments of paper obtained by electrophoresis of the bottom fraction resulting from electroconvection of the fluid from cyst 9. The activity was determined by the test for inhibition of hemagglutination and is expressed as a dilution of the saline extract of the segment analyzed. Fucose and reducing sugars after hydrolysis are the total amounts contained in the segment. The graphical representation of the paper shows the degree of separation of the bands and the manner in which the paper was segmented. An arrow indicates the point at which the original material was placed.

fucose was also concentrated in the strip containing the blood group activity, thus providing evidence that only one fucose-containing substance was present in the cyst fluids. Reducing activity after hydrolysis, however, was localized in 2 distinct areas in the bottom fractions but in only one strip in the top fractions. One of the highest values for reducing sugar was obtained from the strip possessing the serologic activity, as anticipated, while the other peak was secured with material from a strip far removed toward the anode suggesting that this represented an acid polysaccharide. The deep staining bands shown on the strips probably represented protein; this was further substantiated by the fact that the material isolated from these areas as well as material isolated from areas showing blood group activity gave a positive biuret test. The large, intensely staining band closest to the anode was a protein completely unassociated with activity while the area approximately covered by the starting line stained lighter blue and had blood group activ-

(9) D. Aminoff, W. T. J. Morgan and W. M. Watkins, *Biochem. J.*, **46**, 426 (1950).

(10) H. Baer, J. K. Bringaze and M. McNamee, *J. Immunol.*, **73**, 67 (1954).

(11) Some of the anti-A serum was kindly supplied by the Ortho Pharmaceutical Co., Raritan, N. J.

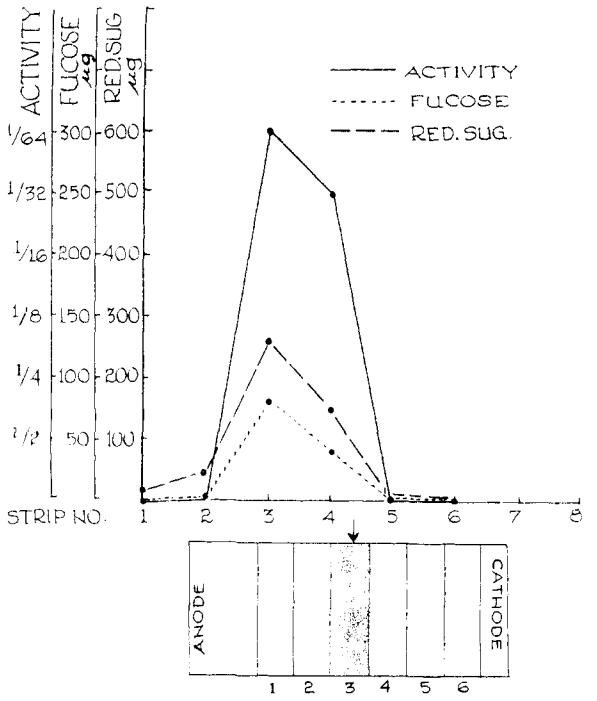

Fig. 2.—Analysis of the segments of paper obtained by electrophoresis of the top fraction resulting from the electroconvection of the fluid from cyst 9. See legend of Fig. 1.

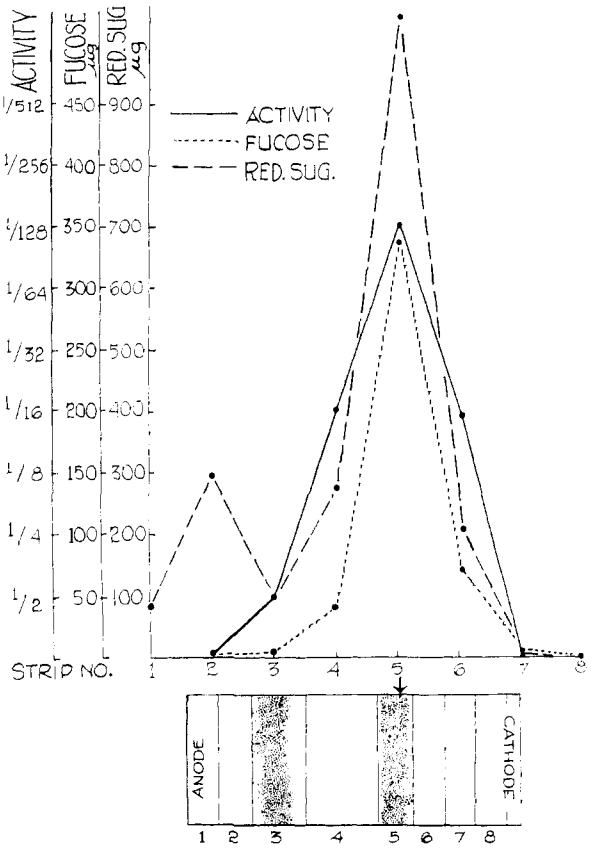

Fig. 3.—Analysis of the segments of paper obtained by electrophoresis of the bottom fraction resulting from the electroconvection of the fluid from cyst 13. See legend of Fig. 1.

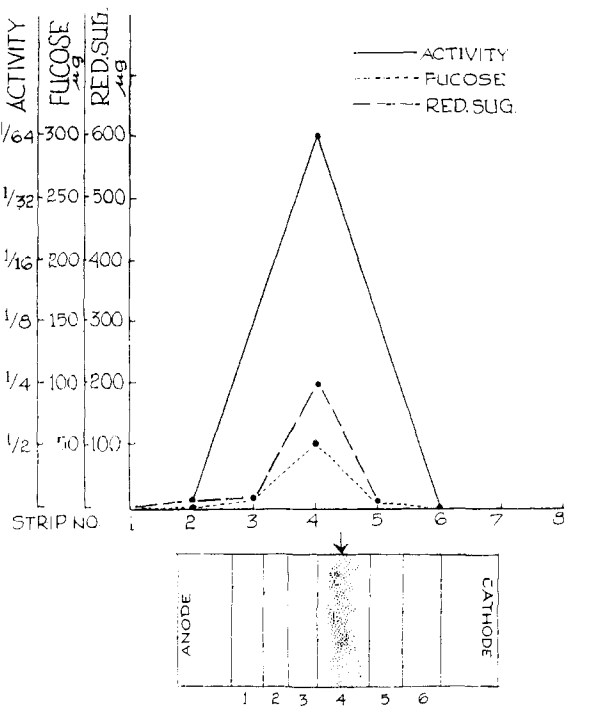

Fig. 4.—Analysis of the segments of paper obtained by electrophoresis of the top fraction resulting from the electroconvection of the fluid from cyst 13. See legend of Fig. 1.

ity, indicating that the blood group activity may indeed be very closely associated with or, more probably, is an integral part of a protein–polysaccharide or polypeptide–polysaccharide complex. Cyst 9 appeared to have a third blue staining band and this too was devoid of activity. It is interesting to note that while the active material showed little if any migration on paper electrophoresis it nevertheless does migrate to some degree since on electroconvection the concentration of the active material is higher in the bottom than in the top fractions. Failure to observe definite migration on paper is probably attributable to a very low mobility in this medium under the conditions used. To obtain high yields, therefore, it is necessary to refractionate the material from the bottom chamber.

To obtain some additional data concerning the homogeneity of the top fractions, a comparison was made of the ratio of fucose to nitrogen as electroconvection progressed. Approximately 50 ml. of cyst 13 fluid, previously dialyzed against phosphate buffer of *p*H 6.9, was placed in the electroconvection apparatus and samples of about 0.1 ml. taken at various times. When it was finally revealed that the top fraction gave only a single band on paper electrophoresis, a 2-ml. sample was removed; about 8 hours later a second sample was removed. At this time the entire top fraction was separated, put into a fresh membrane and replaced in the electroconvection apparatus and the procedure continued for an additional 15 hours. The top and bottom fractions were then separated and analyzed separately; these are fractions 3 and 4 in Table I. Since the data at present indicate that all of the fucose but only a portion of the nitrogen of the cyst fluid is part of the blood group substance, marked

changes of the ratio as electroconvection proceeds would indicate some degree of inhomogeneity. From Table I it will be discerned that the process proceeded until the concentration of fucose in the top fraction had dropped to about one quarter of its original value; the last sample in the table is the concentrated bottom fraction. The ratio for the top fraction was found to fall somewhat as the electroconvection progressed indicating that there may be some contamination by a second substance. The bottom fraction, approximately four times more concentrated than the starting solution had a ratio essentially unchanged from the starting material. None of the ratios even approached values characteristic of materials isolated by the phenol-extraction method. If a nitrogen-containing impurity comprised a large portion of the original cyst 13 top fraction and as a result of continued electroconvection was removed from the top chamber the activity based on the nitrogen content should exhibit a change. The activity of the various fractions was identical indicating that no large portion of inactive nitrogen-containing material was separated from the blood group active substance under the conditions of the experiment.

TABLE I

THE PROPERTIES OF SAMPLES OF CYST 13 FLUID REMOVED FROM THE ELECTROCONVECTION APPARATUS AT VARIOUS TIMES

Sample	N per ml., μg.	Fucose per ml., μg.	$\frac{\text{Fucose}}{\text{N}}$	Min. quantity,[a] as N, necessary for complete inhibition, μg.
1[b]	316	370	1.2	0.0025
2	244	214	0.9	.0025
3	128	92	0.7	.0025
4	1220	1370	1.1	.0025

[a] The inhibition of hemagglutination was carried out with group A erythrocytes and human anti-A serum. The volume of sample employed was determined by its nitrogen content; the final volume in each tube was, however, constant. [b] Samples 1–3 are top fractions, while sample 4 is the final concentrated bottom fraction.

To evaluate and compare the analytical properties and activity of material prepared by electroconvection with that isolated by the phenol-extraction method, the top fractions were dialyzed against distilled water and finally lyophilized. The white solid so obtained was dried to constant weight in a vacuum desiccator over phosphorus pentoxide, and then dissolved in 0.85% sodium chloride solution.[12] The analytical data obtained by the analysis of these solutions as well as some data on the original cyst fluid are given in Table II along with data obtained from a sample isolated from cyst 9 by the trichloroacetic acid, phenol-extraction method and also a sample of A substance isolated from saliva.

Discussion

Paper electrophoresis disclosed that the cyst fluids contained protein and an acid polysaccharide in addition to a substance with blood group activity. Figures 1–4 provide clear evidence that using paper electrophoresis as a criterion of purity, inactive substance can be eliminated from the blood group active fraction by electroconvection under appropriate conditions. Previous studies have established that substances with blood group A, B or O activity are largely polysaccharides but contain some amino acids, presumably as a polypeptide. The present procedure yields a substance with a far higher nitrogen content than substances isolated by other procedures. While it has not been possible to decide unequivocally whether the nitrogen-rich component is part of the blood group active substance or whether the two are present as a mixture, the data point to a single substance, probably a polysaccharide-protein complex. Thus, paper electrophoresis was carried out at *p*H 2.5, 4.9, 6.9 and 8.3 and in each case the activity was associated with a single blue band; the polysaccharide–polypeptide complex isolated by the phenol-extraction method did not stain when placed on paper. Electroconvection was carried out on two cyst fluids and the top fraction in both instances had similar properties (Table II). Analysis of samples removed at various times during the course of electroconvection (Table I) revealed that in none of the samples did the ratio of fucose to nitrogen begin to approach that of material isolated by a phenol-extraction method. The decrease in the ratio may indicate that a second component was removed during the prolonged electroconvection of cyst 13 fluid, but it is interesting to note that the ratio becomes identical with that found for the substance isolated from the fluid of cyst 9 (Table II) suggesting that this might be the correct value for a pure blood group A substance. This procedure, therefore, also indicates that material with a high nitrogen content is an integral part of the blood group substance. It is unfortunate that exhaustion of our sample prevented repetition of this experiment with the fluid from cyst 9.

An indirect piece of evidence has been obtained that also leads to the conclusion that protein is probably part of the blood group substance. While the blood group A and B substances isolated by the phenol-extraction method have been antigenic in man[3,13] and the chicken,[10,14] they have behaved as antigen for the rabbit only after coupling to a protein obtained from *Sh. shigae*.[4–6,15] In contrast to these findings, the substance isolated in the present study has readily yielded specific agglutinins for human red cells of group A and, simultaneously, excellent precipitins for the soluble blood group A substance isolated either by electroconvection or phenol-extraction. Two rabbits were injected twice weekly with 1.0 ml. of an alum-precipitated antigen[10] containing 0.5 mg. of the top fraction of cyst 13 fluid. One week following the last of 6 injections, the animals were bled and the serum examined. While prior to immunization the serum of neither rabbit possessed agglutinins for red cells of groups A, B or O at a dilution of $^1/_4$ or higher, the

(12) On standing for several months in the refrigerator the saline solutions appear to lose some of their activity.

(13) E. Witebsky, N. C. Klendshoj and C. McNeil, *Proc. Soc. Exp. Biol. Med.*, **55**, 165 (1944).

(14) R. Grubb, *Acta Path. et Microbiol. Scand.*, Suppl. 84 (1949).

(15) It has been found that the O (H) and B substances also require coupling to the protein of the Shiga bacillus in order to render them antigenic for the rabbit: E. E. Annison and W. T. J. Morgan, *Biochem. J.*, **52**, 247 (1952); R. G. S. Johns and J. R. Marrack, *J. Hyg.*, **51**, 55 (1953); R. A. Gibbons and W. T. J. Morgan, *Biochem. J.*, **57**, 283 (1954).

TABLE II

Substance	Isolation procedure	N, %	Red. sug.,[a] %	Fucose, %	$\frac{\text{Fucose}}{\text{N}}$	Solids per ml., mg.	Min.[b] quantity for inhibition, μg.
Cyst 9	Original fluid					56[c]	0.25–0.1
Cyst 9 top fraction	Electroconv.	11	24	8	0.7		.025–0.01
Cyst 9 phenol sol.	Phenol-ext.	6		22	4		.1
Cyst 9 phenol insol.	Phenol-ext.	5		22	4		.1
Cyst 13	Original fluid					19	.05–0.025
Cyst 13 top fraction	Electroconv.	10	21	10	1		.025–0.01
Saliva A[d]	Phenol-ext.	5	56	14	3		.1

[a] Calculated as glucose after hydrolysis at 100° for 2 hr. [b] As determined by inhibition of hemagglutination. The values show the range observed on several titrations. All values were determined simultaneously to permit a direct comparison of their activity. [c] The weight of whole cyst fluid represents the non-dialyzable fraction determined by drying a portion of the cyst fluid to constant weight in a vacuum desiccator after dialysis against distilled water. [d] Kindly supplied by Dr. Elvin A. Kabat.

post-immunization sera showed titers of $^1/_{128}$ and $^1/_{512}$, respectively, for cells of group A but none for cells of groups B or O. Precipitins for the homologous antigen and for A substance prepared by the phenol-extraction method were also found in the post-immunization sera. These data will be presented in detail in a future publication. In contrast to these results, the immunization of 4 rabbits with A substance of cyst origin prepared by the phenol-extraction method failed to elicit the formation of either precipitating or agglutinating antibody, thus confirming the work of Morgan and coworkers.

Finally, it has always seemed somewhat strange that blood group active substances frequently appear in several different fractions when isolated from a single source. Thus, phenol-soluble and phenol-insoluble fractions have been highly active[10,16,17] and it has been possible to obtain additional material from water-insoluble residues after prolonged peptic digestion. By the method of electroconvection a single substance is obtained indicating that phenol fractionation may yield an artificial and probably arbitrary separation of the blood group substance into fractions.

A point of at least equal, if not greater, significance is the question of the degree of activity of the various materials. A convenient measure of activity is the minimum quantity of the substance capable of giving complete inhibition of agglutination under a standard set of conditions. The last column of Table II reveals that the substances isolated by electroconvection are considerably more active on a weight basis than those isolated by the phenol-extraction method.

The last column in Table II lists the activity or range of activity found on several determinations. Because of the large error inherent in serial double dilution procedures these figures cannot be determined with great accuracy but if the minimum activity found for each substance is compared, a fairly good estimate of the minimum changes in activity can be given. The original fluids of cyst 9 and 13 showed different amounts of activity on a weight basis, cyst 9 being at least, but possibly more than, twice as active as cyst 13 indicating that the original cyst 9 fluid had more inert impurity than the fluid from cyst 13. The purified materials, on the other hand, had essentially identical activities. A comparison of the various purified substances establishes that substances prepared by the new procedure are at least 4 times more active than substances isolated from cyst fluids or from saliva by the phenol-extraction method.

It may be pointed out that some investigators have described some preparations of blood group A substance isolated from hog stomach lining by the phenol-extraction method to be somewhat more active than human substances isolated by the same procedure. While no attempt was made to compare substances of other than human origin with the materials described in the present work, it is possible that certain blood group A substances of hog origin may approach, in activity these new preparations.

NEW ORLEANS, LOUISIANA

(16) E. F. Annison and W. T. J. Morgan, *Biochem. J.*, **52**, 247 (1952).

(17) E. A. Kabat, A. Bendich, A. E. Bezer and S. M. Beiser, *J. Exp. Med.*, **85**, 685 (1947).