data reported here was reinvestigated, both with the cell-free system and growing cultures of *B. brevis* ATCC 8185. In both cases more than 98% inhibition of peptide and protein synthesis was observed with 10 μg/ml chloramphenicol and 100 μg/ml puromycin. Moreover, the curves for inhibition of biosynthesis with varying concentrations of puromycin and chloramphenicol were virtually identical for both protein and tyrothricin in both types of experiments. The reason for the discrepancy between the present results and those of Mach and co-workers is not presently understood, but further work is in progress to solve this question.

References

Arnstein, H. R. V., and Margreiter, H. (1958), *Biochem. J. 68*, 339.

Ito, E., and Strominger, J. L. (1960), *J. Biol. Chem. 235*, PC7.

Kameyama, T., and Novelli, G. D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 659.

Katz, E., and Weissbach, H. (1963), *J. Biol. Chem. 238*, 666.

King, T. P., and Craig, L. C. (1955), *J. Am. Chem. Soc. 77*, 6627.

Lamfrom, H. (1961), *J. Mol. Biol. 3*, 241.

Nathans, D., von Ehrenstein, G., Monro, R., and Lipmann, F. (1962), *Fed. Proc. 21*, 127.

Okuda, K., Edwards, G. C., and Winnick, T. (1963), *J. Bacteriol. 85*, 329.

Park, J. T. (1958), *in* The Strategy of Chemotherapy, Society for General Microbiology, 8th Symposium, Cambridge, Cambridge University Press, p. 49.

Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.

Uemura, I., Okuda, K., and Winnick, T. (1963), *Biochemistry 2*, 719.

Winnick, R. E., Lis, H., and Winnick, T. (1961), *Biochim. Biophys. Acta 49*, 451.

Winnick, R. E., and Winnick, T. (1961), *Biochim. Biophys. Acta 53*, 461.

Yukioka, M. (1962), *J. Osaka City Med. Center [Osaka Shiritsu Daigaku Igaku Zasshi] 11*, 239.

Zamecnik, P. C. (1962), *Biochem. J. 85*, 257.

Immunochemical Studies on Blood Groups XXX. Cleavage of A, B, and H Blood-Group Substances by Alkali*

GERALD SCHIFFMAN,† ELVIN A. KABAT, AND WILLIAM THOMPSON

From the Departments of Microbiology and Neurology, College of Physicians and Surgeons, Columbia University and the Neurological Institute, Presbyterian Hospital, New York

Received August 7, 1963

Treatment of A, B, and H substances with 0.2 M NaOH in 1% $NaBH_4$ at room temperature yields dialyzable fragments with high blood-group activity. Partial purification of these materials has been accomplished by paper chromatography; active fractions were found in three regions. The most rapidly migrating active fraction from A and B substances was many times more potent in inhibiting A-anti-A or B-anti-B precipitation, respectively, than the most active oligosaccharides previously studied. While further purification and characterization of these fragments is necessary, evidence is presented indicating that they probably contain the entire antigenic determinant.

Success in isolating the antigenic determinants of blood group substances will depend in large measure on finding a procedure which will maintain their integrity but cleave the bonds holding them to the rest of the molecule. Methods involving enzymes (Buchanan *et al.*, 1957; Howe and Kabat, 1953; Iseki *et al.*, 1959; Iseki and Ikeda, 1956; Iseki and Masaki, 1953; Schiffman *et al.*, 1958; Watkins, 1953, 1956, 1960, 1962; Watkins and Morgan, 1955; Zarnitz and Kabat, 1960; for earlier work see Kabat, 1956), acid (Cheese and Morgan, 1961; Côté and Morgan, 1956; Kabat *et al.*, 1946; Kabat and Leskowitz, 1955; Kuhn and Kirchenlohr, 1954; Schiffman *et al.*, 1960; Schiffman and Kabat, 1961; Tomarelli *et al.*, 1954; Yosizawa, 1949; for other earlier work see Kabat, 1956), alkali (Morgan, 1944, 1946; Knox and Morgan, 1954), hydrazine (Yosizawa, 1961, 1962a,b; Yosizawa and Sato, 1962) and resins (Painter, 1960; Painter and Morgan, 1961a,b; Painter *et al.*, 1962) have been used but no thoroughly satisfactory procedure has yet been reported. However, much useful information about the determinants has been accumulated, largely about terminal nonreducing sequences of sugar residues associated with blood group A, B, and H activity (Watkins, 1962; Morgan, 1960; Kabat, 1956) and with cross reactivity with type XIV antipneumococcal serum (Howe and Kabat, 1953; Watkins, 1953; Howe *et al.*, 1958; see also Kabat, 1956). During the course of these studies a considerable number of active and inactive oligosaccharides has been isolated (Côté and Morgan, 1956; Cheese and Morgan, 1961; Painter and Morgan, 1961a,b; Schiffman *et al.*, 1960; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962a; Painter *et al.*, 1962; Yosizawa, 1949; for other earlier work see Kabat, 1956).

With blood group A substance, *N*-acetylgalactosamine is considered to be the terminal nonreducing end of the antigenic determinant (Watkins and Morgan, 1955; Kabat and Leskowitz, 1955; see also Kabat, 1956) and α-*N*-acetylgalactosaminoyl(1 → 3) galactose, a disaccharide more active than *N*-acetylgalactosamine in inhibiting A-anti-A precipitation and hemagglutination is accepted as a disaccharide sequence of the determinant (Côté and Morgan, 1956; Schiffman *et al.*, 1962a). Two active trisaccharides have been reported in which this disaccharide is linked β1,3 (Cheese and Morgan, 1961; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962a) and β1,4 (Cheese and Morgan, 1961) to *N*-acetylglucosamine and more recently Morgan (1962)

* Aided by the National Science Foundation (G-18727), the Office of Naval Research, and the General Research Support Grant from the National Institutes of Health, USPHS. Reproduction in whole or in part is permitted for any purpose of the United States Government.

† Present address: Department of Research Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

TABLE I
BLOOD GROUP SUBSTANCES

Preparation Used	Blood Group Activity	Source	N (%)	Galactose (%)	Hexosamine (%)	Methylpentose (%)	References
Hog mucin Fr. 2	A + H	Hog gastric mucin	6		33	9	Carsten and Kabat (1956)
McDon	A	Human ovarian cyst fluid	5.2	21	33	20	Schiffman *et al.* (1962a,b)
MSS 10%	A	Human *serous* ovarian cyst fluid	7	18	30	16	
MSM 10%	A	Human *mucinous* ovarian cyst fluid	5	19	32	15	
Beach phenol insol	B	Human ovarian cyst fluid	5.2	26	25	21	Allen and Kabat (1959) Schiffman *et al.* 1960
Horse 4 25%	B	Horse stomach mucosa	7.1		28	4.7	Baer *et al.* (1950)
PM phenol insol	B	Human saliva	3.3		20	14	Baer *et al.* (1950)
JS	H	Human ovarian cyst fluid	4	19	25	23	

has reported the isolation of a fucose containing oligosaccharide with A activity.

Galactose is the nonreducing end of the blood group B antigenic determinant and α-galactosyl(1 $\rightarrow$ 3) galactose has been shown to be more active in inhibiting B-anti-B hemagglutination (Painter and Morgan, 1961a) and precipitation (Kabat and Schiffman, 1962) than other disaccharides with a terminal nonreducing galactose. Two trisaccharides have also been isolated from blood group B substance (Painter *et al.*, 1962, 1963) in which this disaccharide is linked β1,3 and β1,4 to *N*-acetylglucosamine.

Blood group H activity is associated with terminal nonreducing α-linked fucosyl residues (Morgan and Watkins, 1953) or methylated D and L fucose derivatives (Springer *et al.*, 1956; Springer and Williamson, 1962, 1963; Kabat, 1962a) and the branched trisaccharide sequence α-fucosyl(1 $\rightarrow$ 4)-[β-galactosyl(1 $\rightarrow$ 3)]-*N*-acetylglucosamine has been implicated in Le^a (Lewis^a blood group) specificity (Watkins, 1962; Watkins and Morgan, 1962) from studies with the oligosaccharides isolated from human milk (Kuhn, 1957, 1958; Egge, 1960; Montreuil, 1960). Lacto-*N*-neotetraose, β-galactosyl-1,4-β-*N*-acetylglucosaminoyl-1,3-β-galactosyl-1,4 glucose, also isolated from human milk (Kuhn and Gauhe, 1962) is the most active oligosaccharide in inhibiting the cross reaction with type XIV antipneumococcal sera of blood group substances exposed to mild acid hydrolysis (Kabat, 1962c; Watkins and Morgan, 1962).

Many years ago Morgan (1944, 1946) reported that treatment of human A, B, and H substances with Na_2CO_3 at *p*H 10.8 at 100° rendered them almost completely dialyzable with loss of serological activity. More gentle treatment with $BaCO_3$ at *p*H 8.5 at 100°, and with changing of the dialysis fluid at hourly intervals (Knox and Morgan, 1954), left only 10% of nondialyzable substances after 32 hours. The composition of this nondialyzable material was essentially unchanged from that of the starting substance. The pooled dialysates were concentrated and precipitated with ethanol at 50, 65, 80, and 90% and then with ether. The 50 and 65% precipitates were active and had the same composition as the starting substance. To date no further information is available about these very interesting fractions. In a Croonian lecture Morgan (1960) reported the isolation of a serologically active trisaccharide containing fucose, galactose, and *N*-acetylglucosamine from an Le^a substance by hydrolysis at *p*H 8.5.

In studying the effect of periodate oxidation on blood group substances it was reported (Schiffman *et al.*, 1962b) that the reducing-sugar values (glucose equivalent using an alkaline ferricyanide procedure) of untreated hog and human A, B, and H preparations were 10–15% and rose only to 50% (Kabat, 1956) on complete acid hydrolysis. Since the high reducing-sugar values were not appreciably lowered after treatment with sodium borohydride, it was concluded that scission by alkali was occurring during the assay. However, reducing-sugar assays of oligosaccharides isolated from blood group substances before and after reduction with sodium borohydride showed that, after conversion of the reducing end to an alcohol, internal glycosidic linkages were stable to alkali. These observations suggested an attempt to degrade the blood group substances with alkali in the presence of borohydride. Such treatment of blood group A, B, and H substances converted the major part of the molecule to dialyzable fragments which have been partially purified by paper chromatography. The products obtained from A and B substances are tens of times more active in inhibiting respectively A-anti-A or B-anti-B precipitation and hemagglutination and are thought to represent the A and B antigenic determinants; similar materials were obtained from an H substance and appear to represent the H determinant.

MATERIALS

Blood-Group Substances.—Table I lists the blood-group preparations used, their specificity (A, B, or H), source and analytical properties. These substances were purified by digestion with pepsin and precipitation with ethanol. The dried ethanol precipitate was extracted with 90% phenol. Hog blood-group substances are soluble in phenol and are precipitated by addition of ethanol to a concentration of 10% by volume. In most instances a second phenol extraction and ethanol precipitation is given. Human substances may either by phenol insoluble or phenol soluble but are precipitated at 10% ethanol from phenol (see Kabat, 1956). For preparations described in earlier studies references are given. The other preparations were made from human ovarian cyst fluids kindly made available by Dr. M. E. Long, Department of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University at the request of Dr. Donald M. Marcus. Of especial interest was the ovarian cyst fluid from MS. Two portions of fluid were obtained.

one serous (MSS) and one mucinous (MSM), and these were prepared separately. In addition, two fractions of Beach blood group B substance obtained after treatment with coffee bean α-galactosidase were studied (Zarnitz and Kabat, 1960; Watkins *et al.*, 1962); these are designated E. T. Beach phenol insol and E.T. Beach 20%.

Some preliminary studies were carried out with precipitate obtained by adding 2 g sodium acetate per 100 ml of crude cyst fluid (MSS) followed by three volumes of ethanol. This dried powder had 14% N, 2.2% galactose, 2.0% methylpentose, and 3.9% hexosamine and is referred to as MSS crude (see Fig. 1).

Antisera.—Type XIV antipneumococcal horse serum H615 (4/15/1939) was obtained through the courtesy of Dr. J. L. Hendry, New York State Department of Health Laboratories, and has been used extensively in previous studies. Human anti-B sera 307 and 310, anti-BP1 262_{4-6} (B absorbed), and anti-AP1 (B absorbed) have been described (Allen and Kabat, 1959). Rabbit glob 897_{1-2} was a globulin preparation from a rabbit antiserum to human A stroma kindly given to us by Dr. C. Howe.

Inhibitors.—Galactinol and α-galactosyl(1 → 3) galactose were gifts from Dr. C. E. Ballou and from Drs. W. J. Whelan, W. Watkins, and W. T. J. Morgan, respectively. Lactose and *N*-acetylgalactosamine were commercial samples.

Methods

Analytical procedures for hexosamine, *N*-acetylhexosamine reducing sugar, galactose, methylpentose, formaldehyde, borohydride reduction, periodate oxidation, paper electrophoresis, and glass fiber paper chromatography were performed as previously described (Schiffman *et al.*, 1958, 1960, 1962a). The procedure for N (Rosevear and Smith, 1961; Schiffman *et al.*, 1962a; Kabat and Schiffman, 1962) was modified as follows: A sample containing 1–4 μg N in less than 75 μl is placed in a 3-ml conical tube and 25 μl of concentrated sulfuric acid diluted one part to twenty is added. The sample is digested in a sand bath, allowing the temperature to rise to 160°, and decolorized with 5–15 μl of superoxol. After the char has cleared the tubes are cooled, and 0.2 ml of water and 0.1 ml of activated ninhydrin reagent are added. The contents of the tubes are mixed, heated for 20 minutes at 95°, diluted with 3 ml of 50% ethanol, and read at 5700 A. The activated ninhydrin reagent consists of 4% ninhydrin in methyl Cellosolve containing 25% 4 M acetate buffer, *p*H 6.5; 0.1 ml of 0.01 M KCN is added to 4 ml of ninhydrin reagent just before use. The temperature of the digestion is lower than that for the usual Kjeldahl and probably not all the N is converted to ammonia, but under the conditions used ammonia and amino acids were found to give equal and maximum color development. Precipitin and precipitin inhibition curves were set up in 3-ml conical tubes over a range of 1–4 μg N by addition of antiserum to tubes containing antigen with or without inhibitor, mixing, and placing in a refrigerator for 1–7 days. In most cases washing of the precipitates could be performed after 1–3 days at 0–4° with results not significantly different from those after 5–7 days. The precipitates were washed twice with 0.5 ml of ice cold saline and analyzed for N as described above.

Hemagglutination and hemagglutination inhibition were performed using a microtitrator (Cooke Engineering Co., Alexandria, Virginia). A 10% saline extract of *Ulex europeus* seeds was prepared and frozen in small portions. An α-galactosidase preparation from Green Santos coffee beans (see Courtois *et al.*, 1958) was prepared by grinding the seeds in a hand coffee mill, extracting for 3 days at 0–4°, filtering through a Buchner funnel (no paper), centrifuging in the cold at 2000 rpm for 1 day, pervaporating the partially clear supernatant fluid to about one-fifth its original volume, and dialyzing in the cold against water for an additional 3 days. Samples of 2–4 ml were frozen and stored. The enzyme was used at *p*H 4.7–4.8 (sodium acetate buffer, 0.001–0.01 M; Arnaud, 1958). One ml of enzyme was used for about 3 or 4 ml of substrate. This preparation was active in splitting melibiose but not lactose.

Experimental and Results

Initial experiments showed that treatment of hog mucin blood group substance in 0.1 N NaOH + 2% $NaBH_4$ for various times at 4° and at room temperature (23°) resulted in the progressive liberation of dialyzable materials. At 4° the reaction was very slow; only 11.6 mg of 30.5 mg of substance became dialyzable after 15 days as compared with 14.4 mg after 2 days at room temperature. After 15 days at room temperature, 22.5 mg had become dialyzable; there was some loss of total recoverable weight, however, since only 4 mg remained nondialyzable. The nondialyzable fractions remaining at varying times were largely unchanged in N, methylpentose, and galactose, but showed lower hexosamine and reducing sugar values. At 48 hours at 4° no change in capacity of the nondialyzable fraction to precipitate anti-A was found, but after 96 hours or more at 4° and after 18 hours or more at room temperature capacity to precipitate anti-A was diminished. The dialyzable fractions were very active in inhibiting A-anti-A precipitation.

Further studies using MSS, a crude alcohol precipitate from an A cyst fluid, were carried out to evaluate the effect of the sodium borohydride on the alkaline cleavage. Samples were treated with alkali with and without borohydride for 2 days at room temperature, neutralized, and dialyzed, and the nondialyzable portions were again treated in the same manner for an additional 5 days. The dialyzable portions from each sample were passed through Amberlite MB-3 and lyophilized. All dialysates were active, the yield of active material was improved by the inclusion of $NaBH_4$, and the N content of the fractions was lower than that using alkali alone. Figure 1 shows the activity of the dialyzable material from crude MSS per microgram sugar in inhibiting A-anti-A precipitation as compared with trisaccharide A_5II, disaccharide A_2Ia, and *N*-acetylgalactosamine. It is evident that the dialyzable material is much more active than the trisaccharide.

In a further experiment a 10-g sample of crude MSS was treated with 100 ml 0.2 M alkali and 1 g borohydride for 7 days at room temperature, neutralized, and dialyzed against successive ten-volume portions of distilled water changing the dialysates after 3 hours and again after 18, 24, 48, 96, 240, and 350 additional hours of dialysis (dialysates 1–7). By conductivity measurements only dialysates 1 and 2 contained appreciable salt, which was removed, after concentrating *in vacuo*, by passage through 11A8 retardion. All samples were lyophilized; analytical properties of all were similar and dialysates 2 through 6 were potent in inhibiting A-anti-A precipitation and represented 3.6 of the 3.8 g of dialyzable material; there was no suggestion that any of the dialysates was more potent.

Based on these initial observations a standardized procedure was applied to the following purified blood group substances (Table 1): MSS 10% (1.6- and 4.0 g

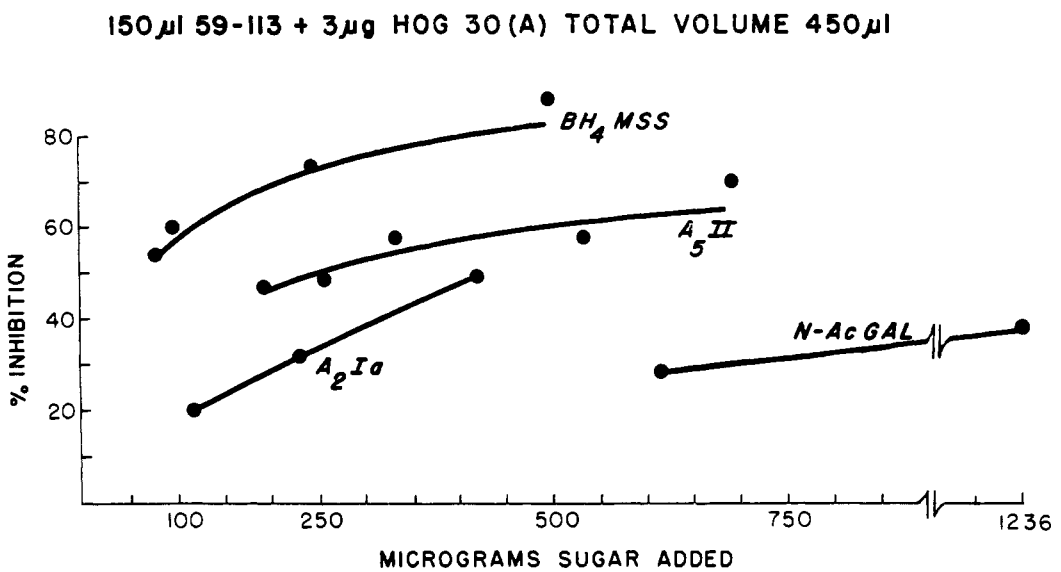

FIG. 1.—Inhibition of A-anti-A precipitation by alkaline borohydride–treated dialysate of human A substance MSS crude as compared with *N*-acetylgalactosamine and the active di- (A_2I_a) and trisaccharide A_3II.

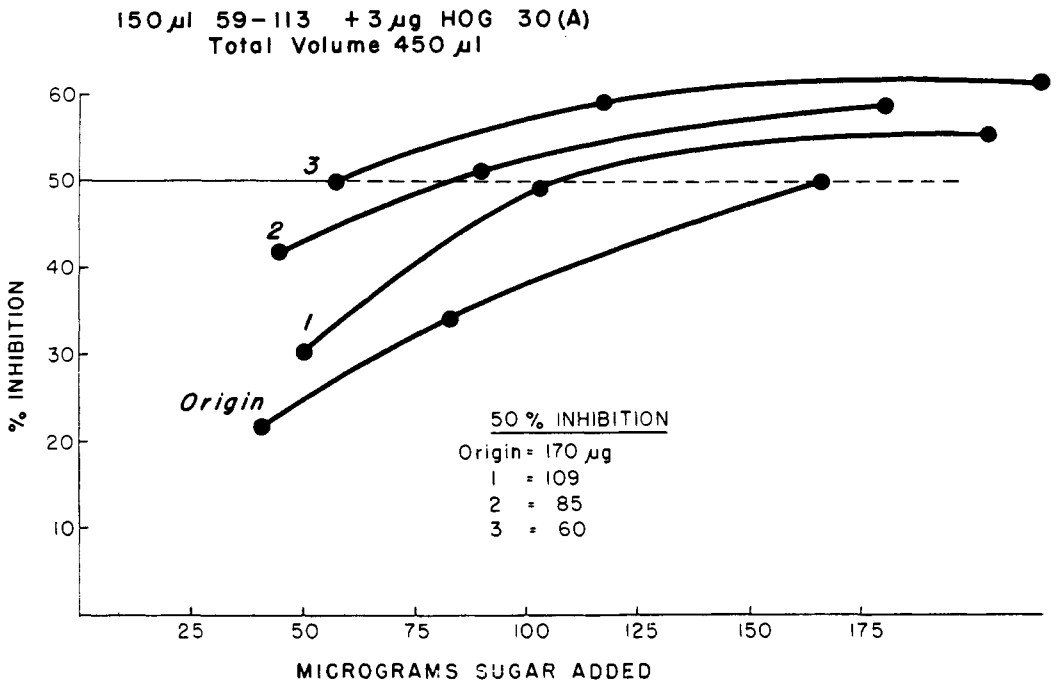

FIG. 2.—Inhibition of A-anti-A precipitation by paper chromatographic fractions of dialysates from alkaline borohydride–treated A substance.

samples in two experiments); MSM 10% (4 g), Beach phenol insol (3 g), JS phenol insol (4 g), and McDon (575 mg). Each sample was dissolved in 0.2 M NaOH containing 1% $NaBH_4$ to a final concentration of 10% and allowed to remain at room temperature for 7 days, neutralized, and dialyzed against ten-volume samples of water. Two changes were made, each after 1.5 hours of dialysis, and the material was pooled and called dialysate 1. Further samples of dialysate were collected after additional intervals of 1, 2, and 4 days (dialysates, 2, 3, and 4). Only dialysate 1 contained appreciable salt and was deionized with retardion. All samples were lyophilized and analyzed. Recovery of methylpentose was quantitative but only 50–75% of the galactose, 63–86% of the hexosamine, and 65–88% of the N were recovered.

Further purification of the dialysates was accomplished by paper chromatography. Preliminary examination on Whatman 3MM paper using propanol–ethyl acetate–water (6:1:3) showed that fractions having the capacity to inhibit A-anti-A hemagglutination were found at the origin and also moved in several regions of the chromatogram up to a region slightly slower than the R_F of lactose. A series of spots could be seen after treatment with alkaline silver. Preparative separation was carried out on strips of S and S 589 green label, 9 in. × 27 in. using 50 mg dialysate per strip. Galactose and lactose were used as guide spots. After 13–15 hours of development allowing the solvent to drip off the end of the paper, the chromatograms were dried and the guide spots were developed with alkaline silver. Papers were cut into 5–7 regions depending upon the positions of the spots. Region 1 included the origin to about one-third the distance to

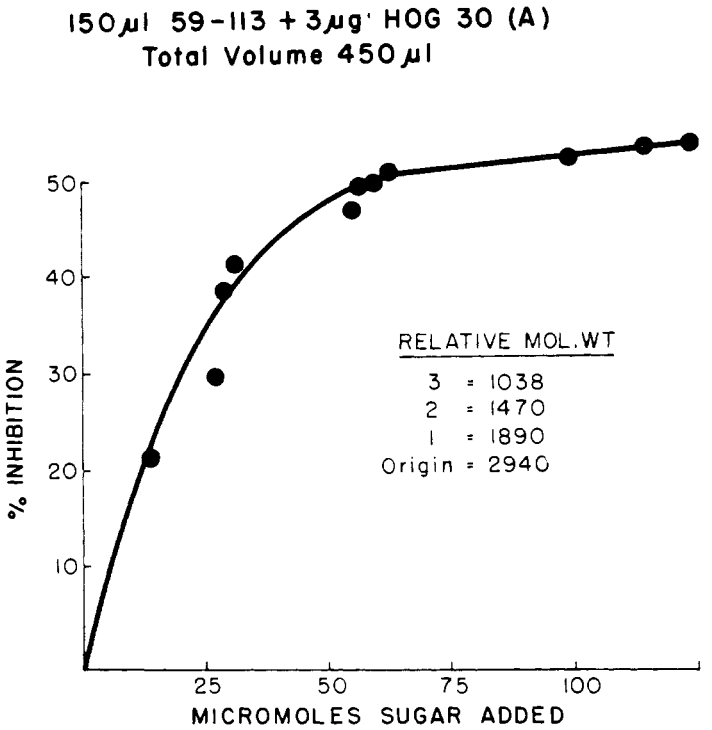

FIG. 3.—Graphical transformation of data in Fig. 2 to a molar basis (see text).

the lactose spot. In some instances the origin was extracted separately. Region 2 represented the second third of the paper from the origin to the lactose spot. Region 3 generally corresponded to a distinct spot with an R_F slightly slower than lactose, region 4 to a spot moving faster than lactose, region 5 between 4 and the galactose spot, and region 6 to the galactose area. These fractions are designated as A_1 to A_6 if obtained from an A substance or prefixed with B or H if obtained from B or H substances. Regions 1 through 3 contained one-half or more of the dialyzable weight. Rechromatography was carried out until a single spot was obtained, two repetitions generally being required. The purified materials were lyophilized, weighed, and analyzed (Table II). Fractions A_3, B_3, B_2, B_4L, and B_4T were brought to this state of purity; B_4 was an elongated spot and was divided; B_4L had the R_F of lactose, and the elongated front portion was called B_4T.

TABLE II
ANALYTICAL PROPERTIES OF PARTIALLY PURIFIED DETERMINANTS AND (5.0 MG/ML SOLUTIONS) RELATED FRACTIONS

	Galactose (µM/ml)	Methyl Pentose (µM/ml)	Hexosamine (µM/ml)	Acetyl Hexosamine (µM/ml)	N (µM/ml)
A_3	3.2	5.3	8.2	6.0	14
H_3	3.5	7.5	4.0	4.4	8.4
H_4	3.1	4.0	4.6	4.8	8.4
B_2	5.2	9.3	4.2	5.1	5.8
B_3	6.6	6.1	5.1	5.4	6.7
B_4L	4.0	4.2	1.7	—	8.9
B_4T	4.0	5.6	3.6	—	4.9

The activity of A_1, A_2, A_3, and of the origin in inhibiting A-anti-A precipitation is shown in Figure 2. Assays were based upon micrograms of sugar added. Fraction 3 with the smallest R_F and presumably the lowest molecular weight was most active. From Figure 2 the weights of sugar required for 50% inhibition were read off, analytical data on the various sugars indicated a minimum molecular weight of about a hexasaccharide for A_3, the molecular weights of the others relative to A_3 were estimated from the amounts required for 50% inhibition, and the data were replotted as in Figure 3 on a molar basis. It is seen that all fractions gave a single smooth curve.

The activity of the purified A_3 as compared with trisaccharide A_5II and *N*-acetylgalactosamine is shown in Figure 4; A_3 gave almost 100% inhibition with as little as 50 mµmoles of sugar. With 59–113 and with another antiserum $1D_{24-25}$ only about 50–60% inhibition was obtained over the range studied. Figure 5 shows that A_3 is also much more potent than A_5II in inhibiting the hemolysis of sheep erythrocytes by rabbit antisera to human A erythrocytes (Forssman activity associated with A substance).

The high potency of B_3 as compared with α-galactosyl(1 → 3) galactose, and galactinol in inhibiting B-anti-B precipitation is seen in Figure 6. With this as well as with a second anti-B serum essentially complete inhibition was obtained.

Fractions A_3 and B_3 were specific in that A_3 did not inhibit the B-anti-B reaction nor did B_3 inhibit the A-anti-A reaction. H_3 did not inhibit either reaction. Assay for H activity with extracts of *Ulex europeus* seeds showed that agglutination of *O* erythrocytes was completely inhibited by 625 µg of H_4 as compared with 25 mg of L-fucose; B_3 was inactive at 2.9 mg, the highest level tested.

The analytical data in Table II, although on impure materials, show that the A_3 determinant contains about 2 moles of methylpentose and almost 3 moles of hexosamine per mole of galactose while B_3 contains about 1 mole of hexosamine and 1 of methylpentose per mole of galactose. In A_3, however, the determination of *N*-acetylhexosamine after complete hydrolysis shows that only about three-fourths of the hexosamine is obtained as *N*-acetylhexosamine, indicating the presence of some *N*-acetylgalactosamine, while in B_3 and H_3 the number of micromoles of hexosamine and *N*-

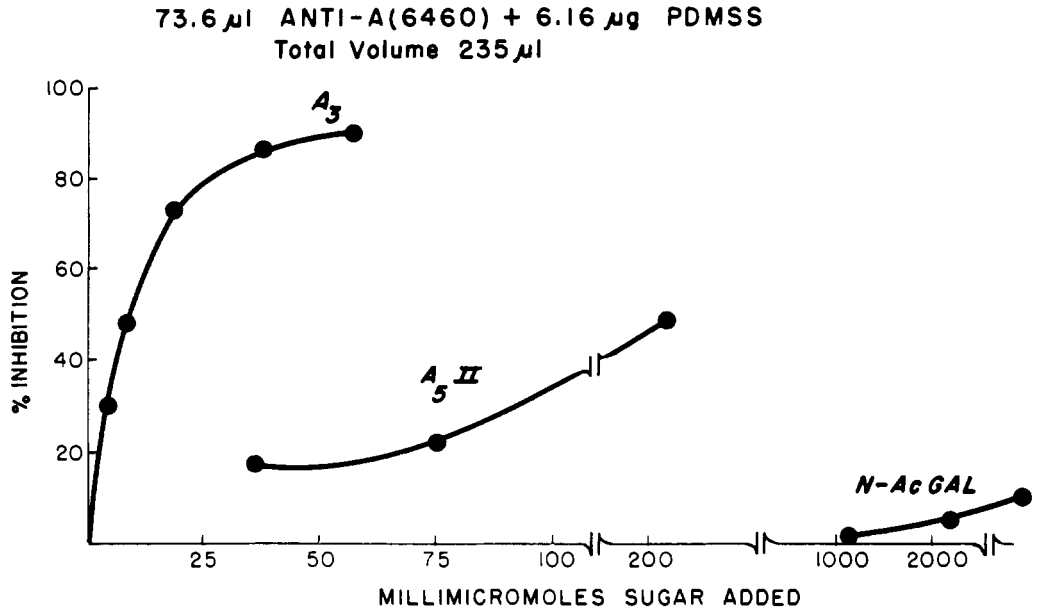

FIG. 4.—Inhibition of A-anti-A precipitation by chromatographic fraction A_3 as compared with trisaccharide A_5II and with *N*-acetylgalactosamine.

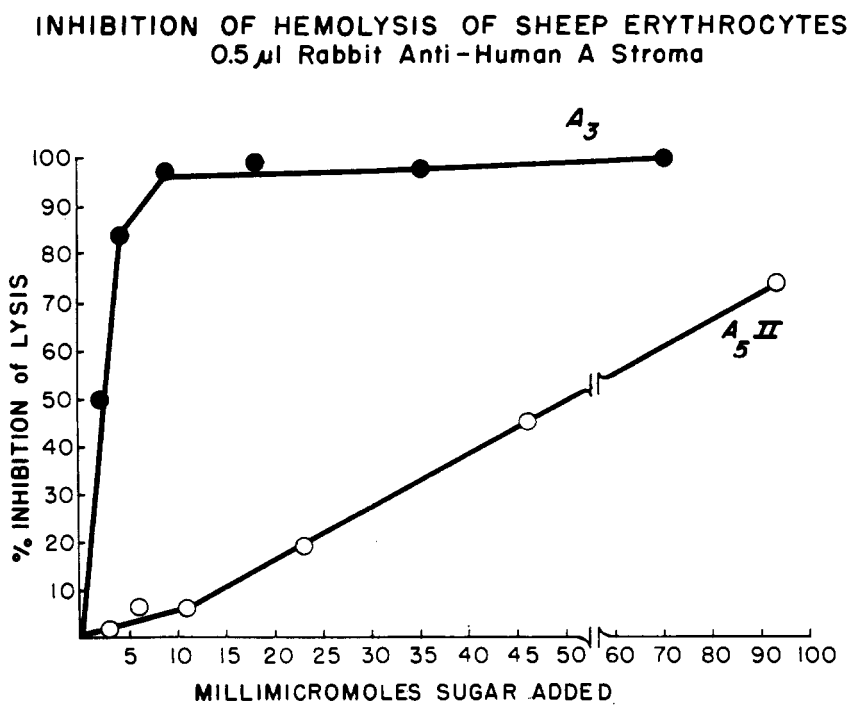

FIG. 5.—Comparison of A_3 and A_5II in inhibiting lysis of sheep erythrocytes by antibody to human A erythrocyte stromata.

acetylhexosamine correspond closely and indicate that *N*-acetylgalactosamine is not present in B_3 and H_3.

The total N content of A_3 shows that 6 moles of nonhexosamine N are present per 8 moles of hexosamine. H_3, H_4, and B_4L also contain considerable quantities of nonhexosamine N. In B_2, B_3, and B_4T, the nonhexosamine N is only about 1–2 µM higher than the hexosamine N. B_4L and B_4T were less active than B_3, B_4L being the least active.

DISCUSSION

The data presented show that alkaline cleavage of human and hog blood group substances from secretions yielded dialyzable fragments representing about 60–80% of the original blood group substances and with activities ten to one hundred times that of the most potent inhibitors thus far reported. This fragmentation at all stages in its course causes little change in gross composition of the nondialyazble materials as compared with the original blood group substance. Similar observations have been made by Gibbons and Roberts (1963) with bovine cervical mucopolysaccharide, a substance closely related to blood group substances (Gibbons, 1959). In the case of the blood group substances the alkaline cleavage is accompanied by loss of 30–50% of the galactose and 14–37% of the hexosamine, probably in the dialyzable fragments; 12–35% of the N cannot be accounted for. Recovery of methylpentose is essentially quantitative, however. The low activity of blood group substances (see Kabat 1956, p. 187, footnote b) prepared by methods involving

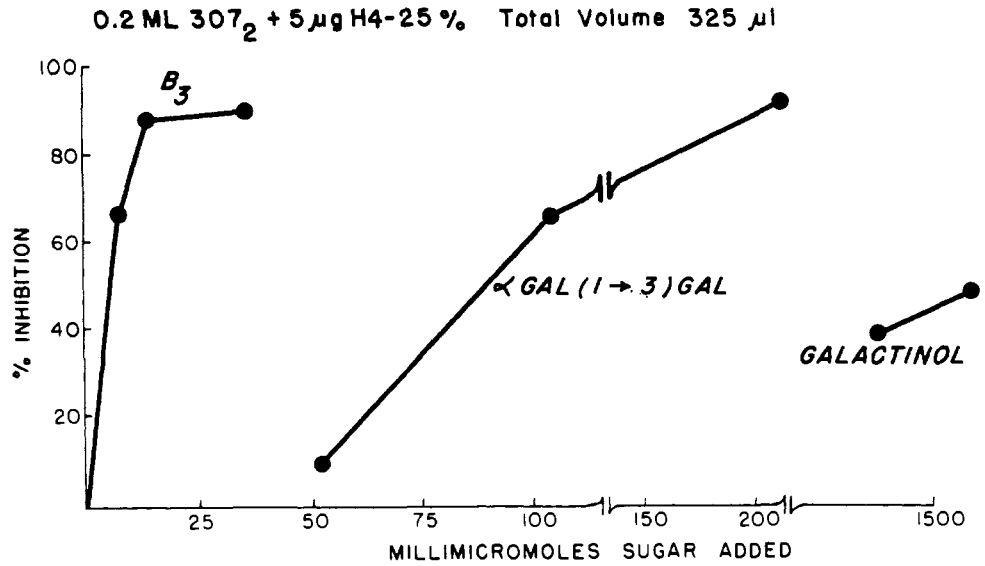

FIG. 6.—Inhibition of B-anti-B precipitation by B_3 as compared with α-galactosyl(1 → 3) galactose and galactinol.

extraction of gastric mucus with 1.5 N NaOH at 15–18° for 1 day now becomes readily understandable.

It was fortunate that the dialyzable materials, with their high *N*-acetylhexosamine and fucose contents and probably because of branching, had very high R_F values on paper as compared with the usual oligosaccharides, since this permitted their fractionation and partial purification. A_3, B_3, and H_3 had an R_F only slightly slower than lactose despite analytical data (Table II) indicating a composition approximating a hexasaccharide. The other materials appear to be of somewhat higher molecular weight; ultracentrifugal examination of A_1 by Dr. H. Rosenkranz using the Archibald method gave an estimate of 1700–2000 for the molecular weight; this would correspond to a unit of 10–15 sugar residues for the slowest migrating fractions. Since this is still very crude, it undoubtedly consists of mixtures of varying molecular weights.

The mechanism of the alkaline cleavage of the blood group substances is not clear. Gibbons and Roberts (1963) suggested that the rapid fragmentation of the molecule made it unlikely that any very extensive polypeptide chain exists in the undegraded material but cautioned that peptide bonds might be made labile by the attachment of sugar residues. The present study shows that paper chromatographic fractionation lowers the N content of the active oligosaccharide fractions, indicating that separation of some of the amino acids from the oligosaccharides has taken place either by splits between carbohydrate and amino acid or of some amino acids from a carbohydrate–amino acid complex. Although the gross analytical composition of the purified A_3 indicates the presence of about 2 moles of nonhexosamine nitrogen per 6 sugar units, assay of a 6 N hydrolyzate of A_3 with an amino acid analyzer through the courtesy of Dr. S. W. Tanenbaum, Dr. E. H. Bassett, and Miss K. Pryzwansky showed the presence of small quantities of all of the amino acids in the original blood group substances. The relation of these amino acids to the sugars is completely obscure, but the failure to find one or two amino acids in stoichiometric amounts in relation to the sugars indicates the need for extensive further purification of these materials.

The high activity of the A_3 fraction as compared with trisaccharide A_3II indicates that this fraction probably contains the entire antigenic determinant of the blood group A substance. All the other fractions could be made to fall on a curve identical with that of A_3 (Fig. 3) by a graphical transformation in which the ratios of the amounts of sugar giving 50% inhibition were read off and assumed to be proportional to their minimum molecular weights on a sugar basis using the empirical composition of A_3 to obtain its minimum molecular weight. This provides a strong indication that the same determinant is reacting in each of these fractions and is merely being diluted with inert sugar material. Were the higher fragments much more active than A_3, it would not have been clear whether A_3 did not contain the complete determinant or whether their increased reactivity was due to their being multivalent with respect to A_3 (see Kabat, 1962b).

The high activity of B_3 and H_4 as compared with oligosaccharides studied previously also indicates that these materials probably also contain the antigenic determinants.

The analytical data in Table II showing that the *N*-acetylhexosamine value of A_3 was less than the total hexosamine on a molar basis provide evidence for the presence of *N*-acetylgalactosamine in the A determinant and are in accord with previous data on the importance of *N*-acetylgalactosamine in A specificity. It is significant that in the B and H fractions no evidence for *N*-acetylgalactosamine was found since the hexosamine and *N*-acetylhexosamine values were equivalent and there appears to be about twice as much galactose in B_3 as in A_3. This accords well with the earlier studies of Gibbons *et al.* (1955) and of Hiyama (1962) on intact blood group substances in which it was found that more galactose was present in B substance than in A substance. It is also in agreement with the findings that on mild acid hydrolysis of A substance more dialyzable *N*-acetylgalactosamine than glucosamine was split off, while with B substance similarly treated only *N*-acetylglucosamine was found in dialysates (Leskowitz and Kabat, 1954).

That the serologically active fractions make up so high a proportion by weight of the blood group substance indicates that there are a considerable number of antigenic determinants in the intact molecule.

REFERENCES

Allen, P. Z., and Kabat, E. A. (1959), *J. Immunol. 82*, 340.
Arnaud, M. (1958), thesis presented to Faculty of Pharmacy of University of Paris.

Baer, H., Kabat, E. A., and Knaub, V. (1950), *J. Exptl. Med. 91*, 105.
Buchanan, D. J., Crumpton, M. J., and Morgan, W. T. J. (1957), *Biochem. J. 65*, 186.
Carsten, M. E., and Kabat, E. A. (1956), *J. Am. Chem. Soc. 78*, 3083.
Cheese, I. A. F. L., and Morgan, W. T. J. (1961), *Nature 191*, 149.
Côté, R. H., and Morgan, W. T. J. (1956), *Nature 178*, 1171.
Courtois, J. E., Anagnostopoulos, C., and Petek, F. (1958), *Bull. Soc. Chim. Biol. 40*, 1277.
Egge, H. (1960), *Bull. Soc. Chim. Biol. 42*, 1429.
Gibbons, R. A. (1959), *Biochem. J. 73*, 209.
Gibbons, R. A., Morgan, W. T. J., and Gibbons, M. (1955), *Biochem. J. 60*, 428.
Gibbons, R. A., and Roberts, G. P. (1963), *Ann. N. Y. Acad. Sci. 106*, 218.
Hiyama, N. (1962), *in* Biochemistry and Medicine of Mucopolysaccharides, Egami, F., and Oshima, Y., eds., Tokyo, Res. Assoc. Mucopolysaccharides, Dept. of Physical Therapy and Medicine, Univ. Tokyo, p 161.
Howe, C., and Kabat, E. A. (1953), *J. Am. Chem Soc. 75*, 5542.
Howe, C., Schiffman, G., Bezer, A. E., and Kabat, E. A. (1958), *J. Am. Chem. Soc. 80*, 6656.
Iseki, S., Furukawa, K., and Yamamoto, S. (1959), *Proc. Japan Acad. 35*, 507, 513.
Iseki, S., and Ikeda, S. (1956), *Proc. Japan Acad. 32*, 201.
Iseki, S., and Masaki, S. (1953), *Proc. Japan Acad. 29*, 460.
Kabat, E. A. (1956), Blood Group Substances, New York, Academic.
Kabat, E. A. (1962a), *Biochem. J. 85*, 291.
Kabat, E. A. (1962b), *Fed. Proc. 21*, 694.
Kabat, E. A. (1962c), *Arch. Biochem. Biophys. Supp. 1*, 181.
Kabat, E. A., Bendich, A., and Bezer, A. E. (1946), *J. Exptl. Med. 83*, 477.
Kabat, E. A., and Leskowitz, S. (1955), *J. Am. Chem. Soc. 77*, 5159.
Kabat, E. A., and Schiffman, G. (1962), *J. Immunol. 88*, 782.
Knox, K. W., and Morgan, W. T. J. (1954), *Biochem. J. 58*, v.
Kobayasi, T. (1941), *J. Biochem. (Tokyo) 34*, 143.
Kuhn, R. (1957), *Angew. Chem. 69*, 23.
Kuhn, R. (1958), *Bull. Soc. Chim. Biol. 40*, 297.
Kuhn, R., and Gauhe, A. (1962), *Chem. Ber. 95*, 518.
Kuhn, R., and Kirchenlohr, W. (1954), *Chem. Ber. 87*, 560.
Leskowitz, S., and Kabat, E. A. (1954), *J. Am. Chem. Soc. 76*, 5060.
Montreuil, J. (1960), *Bull. Soc. Chim. Biol. 42*, 1399.
Morgan, W. T. J. (1944), *Brit. Med. Bull. 2*, 165.
Morgan, W. T. J. (1946), *Biochem. J. 40*, xv.
Morgan, W. T. J. (1960), *Proc. Roy. Soc. (London), Ser. B, 151*, 308.
Morgan, W. T. J. (1962), Presented at IX Congress of International Society of Blood Transfusion, Mexico, Sept. 5–12 as reported to us by Dr. D. Marcus.
Morgan, W. T. J., and Watkins, W. M. (1953), *Brit. J. Exptl. Pathol. 34*, 94.
Painter, T. J. (1960), *Chem. Ind. (London)*, 1214.
Painter, T. J., and Morgan, W. T. J. (1961a), *Nature 191*, 39.
Painter, T. J., and Morgan, W. T. J. (1961b), *Chem. Ind. (London)*, 437.
Painter, T. J., Watkins, W. M., and Morgan, W. T. J. (1962), *Nature 193*, 1043.
Painter, T. J., Watkins, W. M., and Morgan, W. T. J. (1963), *Nature 199*, 282.
Rosevear, J. W., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 425.
Schiffman, G., Howe, C., and Kabat, E. A. (1958), *J. Am. Chem. Soc. 80*, 6662.
Schiffman, G., and Kabat, E. A. (1961), *Fed. Proc. 20*, 67.
Schiffman, G., Kabat, E. A., and Leskowitz, S. (1960), *J. Am. Chem. Soc. 82*, 1122.
Schiffman, G., Kabat, E. A., and Leskowitz, S. (1962a), *J. Am. Chem. Soc. 84*, 73.
Schiffman, G., Kabat, E. A., and Thompson, W. (1962b), *J. Am. Chem. Soc. 84*, 463.
Springer, G. F., Ansell, N., and Ruelius, H. W. (1956), *Naturwiss. 43*, 256.
Springer, G. F., and Williamson, P. (1962), *Biochem. J. 85*, 282.
Springer, G. F., and Williamson, P. (1963), *Vox Sanguinis 8*, 177.
Tomarelli, R. M., Hassinen, J. B., Eckhardt, E. R., Clark, R. H., and Bernhart, F. W. (1954), *Arch. Biochem. Biophys. 48*, 225.
Watkins, W. M. (1953), *Biochem. J. 54*, xxxiii.
Watkins, W. M. (1956), *Biochem. J. 64*, 21 p.
Watkins, W. M. (1960), *Bull. Soc. Chim. Biol. 42*, 1599.
Watkins, W. M. (1962), *Immunology 5*, 245.
Watkins, W. M., and Morgan, W. T. J. (1955), *Nature 175*, 676.
Watkins, W. M., and Morgan, W. T. J. (1962), *Vox Sanguinis 7*, 129.
Watkins, W. M., Zarnitz, M. L., and Kabat, E. A. (1962), *Nature 195*, 1204.
Yosizawa, Z. (1949), *Tohoku J. Exptl. Med. 51*, 51.
Yosizawa, Z. (1961), *Biochim. Biophys. Acta 52*, 588.
Yosizawa, Z. (1962a), *J. Biochem. (Tokyo) 51*, 1, 145.
Yosizawa, Z. (1962b), *in* Biochemistry and Medicine of Mucopolysaccharides, Egami, F., and Oshima, Y., eds., Tokyo, Res. Assoc. Mucopolysaccharides, Dept. of Physical Therapy and Medicine, Univ. Tokyo, p. 117.
Yosizasa, Z., and Sato, T. (1962), *J. Biochem. (Tokyo) 51*, 233.
Zarnitz, M. L., and Kabat, E. A. (1960), *J. Am. Chem. Soc. 82*, 3953.