UMBILICAL CORDS AS A SOURCE OF BLOOD GROUP "A" SUBSTANCE

by

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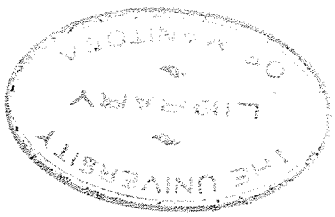

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INTRODUCTION

The Winnipeg Children's Hospital, because of its intimate association with, and deep interest in, the transfusion of blood, desired to have certain complications, incidental thereto, overcome. Accordingly, at its request, the following research project was commenced to deal with one phase of the problem concerning the adverse effects of single, or repeated, transfusions of certain inhomologous blood, on the recipient.

Human blood has been classified into four common blood groups known as A, B, AB and O. This division was based upon the presence or absence of the agglutinable substances, or agglutinogens, designated as A and B, within the erythrocyte. Erythrocytes containing A substance are classified as group A; those containing B substance, as group B. The serum or plasma from group A blood contains the isoagglutinin, anti-B, a globulin which can cause the isoagglutination of B cells. Conversely, group B plasma or serum contains the isoagglutinin anti-A, which can agglutinate A erythrocytes. (The term <u>iso</u>agglutination refers to agglutination occasioned by the intermixing of cells and serum of individuals of the <u>same</u> species; similarly, <u>iso</u>agglutinin). Group AB blood contains both A and B substances, consequently, its serum or plasma contains neither anti-A nor anti-B. Group O erythrocytes were considered to be cells lacking the A and B agglutinogens and were recognized by the absence of these factors, rather than by the possession of a specific and characteristic agglutinogen. It is now believed that O blood does possess a specific O factor analogous to the A and B agglutinogens (63), and the occurrence of an isoagglutinin, causing the agglutination of O cells, has been reported by Schiff (69), and Landsteiner and Levine (54). These serological differences in blood are determined solely by heredity (77).

Because O cells are not agglutinated or hemolysed by any normal human serum, a person having blood of group O has been termed a "universal" donor. This concept was developed by Ottenberg (65), who believed that the presence of the isoagglutinins anti-A and anti-B in O blood could be neglected due to the high dilution of the transfused blood, which would prevent any agglutination or hemolysis from being significant. In practice, however, evidence has been accumulated which indicates that this theory does not apply in all cases. In general, reaction of group A and B recipients has been relatively more frequent when transfusion of O blood was made, than when homologous blood was transfused. These reactions varied in type and severity from an undue reaction rate to fatal hemolysis (47). Reactions due to erroneous blood group determinations were excluded in the survey.

At present, it is believed that the concentrations of the isoagglutinins in O blood may vary greatly, and that reaction occurs whenever the blood of the universal donor has anti-A and anti-B present in concentrations sufficiently above normal to react severely with the cells of the recipient. It is significant that in New York State the O blood to be used as universal blood must first be titrated against group A and group B bloods, to determine its anti-B and anti-A properties (47, 81).

The occasional presence of excessive amounts of the isoagglutinins is the only strong objection to the universal use of O blood for transfusion purposes. It has now been found that "conditioned" universal blood — that is, O blood in which an adequate reduction, or complete elimination, of anti-A and anti-B has been carried out by the addition of suitable amounts of the A and B agglutinogens — may be used with safety, without significant hemolysis or isoagglutination of the recipient's blood (47). The use of O blood, so treated, eliminates the necessity of typing and cross-matching as

standard procedure, in emergencies.

A and B substances have been produced from animal sources, chiefly commercial peptone and pepsin, but these have been found not identical with the A and B substances from human sources (4, 6, 8, 14, 15, 38, 64). Moreover, there is an associated protein, or amino acid, moiety which is potentially dangerous to humans. The protein moiety of A and B substances from human sources is much more, if not entirely, harmless to humans.

Several human sources of the blood group substances have been reported hitherto. These include the erythrocytes of group A and group B blood (11); semen from individuals of blood group A or B (54, 83); gastric juice (79), which could be obtained only in very small amounts; the rarely obtainable, ovarian cyst fluid (61); saliva (52), which, in consistently large amounts, would be extremely difficult to obtain; urine (20), which contains the agglutinogens in excessively high dilution, and, meconium (68), which would be difficult to obtain in quantity.

Meconium is the first intestinal discharge of the new-born, and represents material accumulated during fetal life. As well, it has been found that the isoagglutinins in the blood of the new-born are those which have been transmitted from the mother through the placenta into the fetus' circulation (77). This was confirmed by Wiener and Silvermann (78) who made parallel titrations of the isoagglutinins in maternal and umbilical cord blood and found the ratios to be constant. Besides this, a polysaccharide similar to the blood group substances has been isolated from umbilical cord (55, 56, cf. 57). These findings suggested that the blood group substances might be obtainable from umbilical cords, a material which, if found to be a feasible source, could readily, and at little cost, be obtained in quantity in almost any large community. Accordingly, this

research was undertaken with these two aims: first, to determine whether or not the A agglutinogen was present in the umbilical cords of infants of blood group A; and, second, to find or develop a suitable method for its extraction from this source.

REVIEW OF THE LITERATURE

Since 1900, when Landsteiner first reported his observations on the serological differences existing between bloods of normal individual human beings (cf. 48), the cause of this differentiation has been sought diligently.

Sources of the Agglutinogens. The material responsible was first isolated (11) from blood, by ethanol extraction of the erythrocytes. However, blood has proved to be a difficult and unrewarding source (74). All three agglutinogens have been found in the stroma of the erythrocytes of the various blood groups (19, 29). Water-soluble forms of the blood group substances have been obtained from erythrocytes by washing with normal saline (27) or with distilled water (28). The most recent publication regarding human blood as a source (74), reported that 25 mgm. of agglutinogen per 500 ml. blood was the maximum obtained.

Spermatozoa were the first source material found, other than blood. These were shown (54, 83) to absorb, almost completely, the isoagglutinins from sera with a specificity paralleling that of the erythrocytes. This absorption was believed to be due partly to the fluidal part of the semen, as well as the spermatozoa (83).

It soon became apparent that the blood group substances were present in a large number of the tissues and secretions of human and animal bodies, including saliva and vaginal fluid (83); milk, bile, urine, gastric and duodenal juices (12); liver, brain, kidney and spleen (70).

In 1932, Putkonen (67) reviewed the sources of the blood group substances found at that time, and concluded that, of all the sources tested, gastric juice and saliva were the most potent. The study revealed that the secretion of the antigens and antibodies was not universal, certain

individuals being found non-secretors.

Urine was one of the first sources to be closely studied (20). Group specific carbohydrates were obtained from urine from humans of blood groups A, B and O (21, 22). The material from group A urine was much more active than the carbohydrates from groups B or O (21). More recently, it has been reported (36) that two different A substances can be obtained from human urine.

A detailed and systematic study of glycoproteins from various sources, both human and animal, by Meyer, Smyth and Palmer (57), revealed the presence of a neutral mucopolysaccharide in hog gastric mucosa, which was regarded as being the most active blood group preparation then isolated. An acid polysaccharide, isolated at the same time, was found to be much less active. Similar neutral polysaccharides were subsequently isolated from commercial peptone (25) and pepsin (51, 52).

While working with hog gastric mucin, Morgan and King (62) devised a widely used method of purification, based on the assumption that the agglutinogens were easily degraded, labile complexes similar to other tissue and cellular antigens (58) and that to obtain undegraded samples, extremes of pH and temperature had to be avoided. This concept has been shown to be erroneous (15, 23, 35, 42). Nevertheless, using this method (62) an extremely pure, although electrophoretically inhomogeneous (42), A substance was obtained from hog gastric mucin (62). This same method (62) was used to purify A substance isolated by the peptic and autolytic digestion of individual hog stomach linings (10). Inactive mucopolysaccharides were obtained concurrently, but further investigation (4, 9) suggested that these were really the O specific mucoid.

Holzmann and Niemann (31) obtained very pure and highly uniform

samples of hog mucin A substance by ethanol fractionation and ensuing concentration of the material by electrodecantation. Yoshizawa (84) also obtained extremely potent samples from the same source by electrodecantation after NaOH digestion and ethanol precipitation.

Horse stomachs were shown to be a rich source of A (7) and of B substances (44). Glandular portions were found to be better sources than non-glandular portions, and the stomach lining better than the muscular walls (44). Substances exhibiting A, B or O activity, as well as inactive material, were isolated from individual bovine stomachs (8) by the method of Morgan and King (62). This bovine B substance was shown to have only 1 - 5% of the activity of B substances from human saliva and horse stomachs. The abomasus of cattle has also proved to be a source of A substance (35).

Isoagglutination inhibition tests (cf. refs. 14, 43, 62, 75) indicated that an A specific, carbohydrate-like material isolated from horse saliva (49, 50) was highly active. Later, substances, mucoid in nature, which possessed strong group-specific serological characters, were isolated from human saliva (52).

Small amounts of B and O substances have been obtained from gastric juices of donors of the respective blood groups (15, 79, 80). However, such small amounts of the agglutinogens were present that the homogeneity, or even the high purification, of the final product was not established.

Further investigation of human secretions disclosed that pseudomucinous ovarian cyst fluids were excellent sources of A and O substances (61). Specimens were found that contained 100 times as much A substance per ml. as active group A saliva. O fluids, also, were shown to be more

active than corresponding salivas. The O substance has been isolated from this source in a pure form (63) using a modification of the method of Morgan and King (62). B substances were also obtained from these fluids but no really potent specimen was encountered (61).

The group-specific activity of meconium has been known for many years (82), but only recently has it been proven a practical source of a potent and accessible form of the human blood group substances (68).

A water-soluble amino-acid-polysaccharide complex, which showed A specificity, was extracted from several sources by Bray, Henry and Stacey (14) using a modification of Sevag's method of deproteinization (71).

In a detailed survey of all previously reported methods (16), the method of Landsteiner and Harte (53), with suitable modifications, was judged to be the most convenient method of obtaining undegraded material, which, in yield, purity, and potency, was equal, or superior, to the other methods examined. More recently, it has been claimed (23) that an adaptation of the Folin-Wu method gave equivalent results much more readily, but that the method of Goebel (25) yielded materials of the highest purity.

Properties of the Agglutinogens. The nature of the blood group specific substances has been studied by many investigators. The original material used for the isolation seemed to play an important role in the inquiries.

The earliest investigations (12, 13, 21, 22) established the blood group substances as essentially protein-free, lipid-free polysaccharides. Phosphorus and sulfur have been reported present in various concentrations (29, 41) although some preparations (6) contained none. A large molecular weight was suggested by the failure of the A substance to dialyse (12). Nitrogen was found in the A substance from peptone (13) and from

human urine (20); this was taken as indicating the presence of protein (51). Hydrolysis of the human urine A substance disclosed the presence of galactose and an aminohexose (21), later identified as glucosamine (51). Subsequent investigations (22) suggested the presence of pentosans, and showed that the removal of acetyl groups from A substance yielded a serologically inactive product which could be reactivated by acetylation. Further research indicated that the acetyl groups were attached through the amino groups of the hexosamine (51).

Sevag (71) believed that the polysaccharide moiety of bacterial cells, egg albumin, blood serum, and such, was bound to the accompanying protein through adsorption, rather than chemical bonds. Modern chemical theory (17) no longer distinguishes between adsorption and chemical binding since identical types of forces are operating. The neutral group A specific polysaccharide from hog gastric mucin was considered by Meyer et al. (57) to be composed of N-acetylglucosamine and galactose. Because only part of the nitrogen could be removed, these workers concluded that the natural carbohydrate was firmly bound to the nitrogenous moiety, which existed as a polypeptide chain. Alkaline or enzymatic hydrolysis was believed to rupture this chain, resulting in the observed decrease in nitrogen content. Co-workers (52), and others (14), also reported amino acids in their most active preparations, but were hesitant as to whether or not they were actually part of the group specific material (6). Purified A substance from hog gastric mucin was reported to contain more than one-third of the total nitrogen as amino acids (53, 60). Similar results were obtained using A substance from human saliva (52). These findings established the A substance as an amino-acid-polysaccharide complex.

It is now known that all of these earlier preparations were

inhomogeneous (cf. 45) and that the A substance isolated from commercial hog mucosa preparations are contaminated with one other mucoid at least (6, 32). This has been illustrated with ovarian cyst O substance (63) and the human urine A substance (36) although the former has now been obtained in an electrophoretically homogeneous state (6, 46).

Spectrophotometric studies of A and O substances indicated that both contained approximately 18% of N-acetylglucosamine and exhibited reducing properties equivalent to 4 - 5% of glucose (63). The A substance was observed to contain an unknown impurity absorbing maximally at 2600 Å (33). These contaminants, subsequently proven to be nucleic acids (34), are entirely absent in pure A substance.

Methylation studies (14) corroborated prior reports (58) that L-fucose and D-mannose were present in A substance. Methylated derivatives of D-galactose and D-glucosamine were also identified (14). The isolation of 2, 3, 4 - trimethyl - α - methyl - L-fucoside and of 3, 4, 6 - trimethyl - α - methylglucosaminide hydrochloride from the esterified products from the acid hydrolysis of A substance (14) indicated that fucose and glucosamine were present as non-reducing terminal units. Later investigations (37, 39), which confirmed the end position of the fucose, suggested that the fucose was only weakly bound to the rest of the molecule. A similar conclusion was reached (5) after chromatographic analysis of the A substance.

The presence of mannose became controversial when investigators (9) isolated L-fucose (as the diphenylhydrazine), D-galactose (as the methylphenylhydrazone), and D-glucosamine (as the hydrochloride), but no mannose from hog stomach A substance. Furthermore, the examination of highly purified, but evidently inhomogeneous (cf. 1), peptic A substance

by filter-paper partition chromatography (66) indicated the presence of fucose, galactose and glucosamine, and a fourth, but unknown, component. No indication of the presence of mannose was observed, and, although chondrosamine was looked for, none was found. Similar results were reported in subsequent investigations (1) except that these strongly suggested that chondrosamine was present.

The information gathered from the oxidation studies of the A substance with periodic acid settled this controversy. These results (2) established the presence of D-galactose, L-fucose, N-acetylglucosamine, and N-acetylchondrosamine. An ensuing publication (6) suggested that the A substance was a polymer having as its basic unit one fucose, one galactose, and two N-acetylhexosamine residues. Sedimentation and diffusion data (46) indicated a molecular weight of approximately 280,000, which implied that there were about 280 units. These estimations have been revised since then and the fucose, the galactose and the hexosamines were proven to be present in equimolecular proportions (3). In order to conform to the chemical properties evinced by the periodate oxidation the smallest feasible unit was deduced to be composed of four molecules of each of the four sugars.

Although there are at least eleven amino acids in the amino acid moiety (6) results indicated that the thio- and aromatic amino acids were absent or present only in traces. Threonine and serine were proven (3) not to be present as terminal residues in the protein moiety. Recent studies of the amino acid moiety by mild acid hydrolysis indicated that aspartic acid was present in amounts equal to the sum of all other amino acids present (6, 76).

Evidence has been uncovered which certain investigators (59, 73)

interpreted as an implication that the two characters of the A substance, one affecting hemolysis, the other isoagglutination, were quite separate in the natural material. The individuality of these characters was emphasized (5) when, on dialysis of the mild acid hydrolysates of A substance, only one fraction could be exhibited in the dialysate. This was the moiety responsible for isoagglutination; the non-diffusible fraction was proven to be responsible for hemolysis. Chromatographic analysis (5) showed that the latter contained very little of the original fucose, thus proving that the pentose was not closely linked with the structure associated with hemolysis inhibition. Other investigators (42) noted that the A activity was much more stable than other precipitin characters and suggested that the various serological characters depended upon various portions of the molecule.

It has been reported that two forms of A substance, one acid soluble, the other acid insoluble, can be obtained from the human urine (36) and the hog mucin (32) A substances, although this has not been demonstrable with ovarian cyst A substance (6).

Whereas it has been amply illustrated (38, 64) that the chemical composition of the blood group A substance varied with the species of the source animals, variations within a species have been observed. In general the A substance from non-human sources was laevorotatory (14, 15), while that from human sources was dextrorotatory (6, 15). However, the A substance from human saliva has been shown to contain laevorotatory mucopolysaccharides (41). The activity of the blood group substances has shown great variation amongst individual sources of the same species (10, 41). Diversities have been reported in the precipitin reactions of the A and O substances from individual hog stomach linings (39), and the A

agglutinogens from human saliva, stomach (40) and amniotic fluid, with Type XIV antipneumococcal horse sera, although the preparations were of equal serological purity and capacity to precipitate human anti-A (10, 39). These results have not, as yet, been explained.

The preceding discussion has endeavored to point out the progress made respecting the composition and structure of the blood group substances, now regarded as well-defined polysaccharide-amino-acid complexes, although characterization is by no means complete. However, with the increased understanding of these agglutinogens, improved methods of extraction and purification (cf. post) have been developed, and products of constantly increasing purity and homogeneity so obtained. Mild acid hydrolysis has thrown much light upon the content and structure of the amino acid moiety and on its effect on the specificity of the blood group substances (76). Recently, organic analysis, paper chromatography, and electrophoresis have been used to great advantage. Although, at present, the intra-special serological variations have proved somewhat of an enigma, these diversities are being viewed from the standpoint of biochemical genetics (60). Concerned, as it is, with the innate differences between individuals, this new field of study, through an immunochemical approach to the problem of determining the precise differences between these closely related gene products, may ultimately express, in chemical terms, the specific action of the blood group substances in man.

EXPERIMENTAL

PRINCIPLES OF THE METHODS

The solubility of the blood group specific substances in water and in phenol has been utilized in the many different methods of extraction used to obtain the agglutinogens. Normal saline was used, with some success, to extract these materials from blood (27). However, it was found (28, 29) that simple water extraction was more suitable, since the intracellular material was made available due to hemolysis.

These methods were insufficient when extraction was attempted from human or animal tissue because the water could not penetrate the cellular membrane adequately. To overcome this difficulty, some investigators (72) ruptured the cells by alternate freezing and thawing, using liquid air as the refrigerant. Besides the mechanical destruction of tissue, the low temperature was believed to result in the cleavage of loosely bound materials — especially chemically dissimilar substances like protein and carbohydrate — and to produce a physical change in the protein, in such a way that it was either difficultly soluble in water, or readily precipitated from solution (71). Recently, the thermolability of the agglutinogens having been confuted, hot water has been used (14) to extract the group specific substances from cellular tissue.

Alkaline (83) or acid hydrolysis (50, 52, 57) has been shown to aid in the extraction. Extensive simplification of protein-carbohydrate complexes, and almost complete disintegration of cell membranes, has been effected by peptic digestion (7, 10) of the source material in aqueous suspension, or by autolysis (10) where glandular material is employed.

The use of 90% (w/v) phenol (62) has been highly successful in extracting the agglutinogens from tissue because much of the cellular protein, as well as the blood group specific mucopolysaccharides, is soluble in it. This ability to dissolve protein assisted greatly the penetration of the cells by the solvent.

In all methods, the blood group substances are precipitated from solution by the addition of ethanol, acetone, or glacial acetic acid. The protein content of the mucopolysaccharide is diminished either before or at the time of the precipitation.

Sevag (71) used a chloroform-amyl alcohol mixture to coagulate the dissolved protein. It has been suggested (71) that many of the observed effects of the chloroform are due, probably, more to the influence of temperature, pH, and other conditions of the reaction than to the reagent itself. Precipitation of the proteins by the addition of sodium tungstate in the presence of dilute sulfuric acid has been demonstrated (23) to be a rapid and effective method of deproteinization. These deproteinizations are carried out before precipitation; when the blood group specific substance is obtained by ethanol fractionation, protein separation occurs simultaneously with the precipitation. This separation of the pure specific polysaccharides from accompanying inert dissolved protein or protein degradation products is accomplished through fractional precipitation with ethanol on the assumption that the concentration of ethanol sufficient to precipitate quantitatively the agglutinogen is insufficient to precipitate the associated nitrogenous material (24).

METHODS OF EXTRACTION

Since a large number of different materials, similar in amount

and appearance, had to be transported from the University to the Children's Hospital, and handled by more than one person, a system of code numbers was instituted. Each method was assigned a code number by which, in association with one or more distinguishing letters and numbers, every preparation obtained by that method, or by a variation thereof, was identified. Lower case letters were used in the code numbers to indicate the blood group of the source material when blood group B or O umbilical cords were employed. Thus 8-b material was obtained from group B cords by the method assigned the code number 8. No such indication was made where group A cords were used, or where group A specific material was anticipated.

Where it was feasible, code numbers suggestive of the method of preparation, were assigned. The code number 5-PT, for example, was allotted to a substance obtained from group A cords by a variation from the method given the code number 5, after both peptic and tryptic (pancreatic) digestion.

The following are the methods used to extract the agglutinogens from umbilical cords:

METHOD I (Code number, 3): Umbilical cords of infants of blood group A were dehydrated by storing under acetone for twelve to fifteen days or until used, as described by Meyer and Palmer (56). After being scrubbed free of all adhering blood, the cords were dried in an oven at 37°C. until dry and brittle, and then chopped to a coarse powder in a Wiley mill.

These cords (dried weight, 12.25 gm.) were then deproteinized according to Sevag's directions (71). The cords were ground into a thick paste with 12 ml. of distilled water in a large mortar. This paste was transferred to a litre Erlenmeyer flask and mixed with 240 ml. of dis-

tilled water, 72 ml. of chloroform and 24 ml. of amyl alcohol, after which the mixture was shaken for sixteen hours. The contents were then centrifuged for twenty minutes at 3000 R.P.M., during which time they separated into three very distinct layers. The top layer, a slightly viscous, opalescent solution, made up about one half of the total volume. The middle layer, constituting approximately one quarter of the total volume, was solid umbilical cord material. The bottom layer was a yellowish, translucent, viscous solution, containing the chloroform and amyl alcohol. The top layer was poured off and preserved. The two lower layers were mixed together and deproteinized twice more as described above.

The supernatants were combined and a portion transferred to a large porcelain evaporating dish 20 cm. in diameter, so as to form a thin layer in the bottom of the dish. This was then placed in an oven at 37°C. and the aqueous extract, in this way, evaporated to dryness. The residue was then dissolved in 100 ml. of distilled water and shaken for sixteen hours with 40 ml. of chloroform and 4 ml. of amyl alcohol. After centrifuging for twenty minutes at 3000 R.P.M., the supernatant solution was filtered through Whatman's #42 filter paper. The filtrate was evaporated to dryness at 37°C. as before, in order to remove all the chloroform and amyl alcohol. The residue was dissolved in 50 ml. of distilled water and mixed with 250 ml. of 95% ethanol to precipitate the agglutinogen. The precipitate was collected on Whatman's #42 filter paper, transferred to a porcelain evaporating dish, and dried over P_2O_5 *in vacuo*. The final product (Code number, 3) weighed 0.0718 gm.

METHOD II (Code number, 4): Following the method of Meyer and Palmer (56), 21.22 gm. of umbilical cords of infants of blood group A, dried and

ground up as in Method I, were treated with one 200 ml. portion, and four 100 ml. portions of 90% (v/v) acetic acid, to remove all blood from the cords. Each portion of acetic acid was drawn off with a sintered glass filter stick before the next portion was mixed in. The residue was washed free from acid by suspending in three 100 ml. portions of 95% ethanol.

The material was then suspended in 200 ml. of distilled water and refrigerated overnight. The supernatant aqueous solution was decanted and preserved. This process was repeated five times. Each extract was tested with Molisch solution. The sixth extract failed to give a positive Molisch test and was discarded.

The five supernatant solutions were combined (total volume, 850 ml.), made acid to litmus with 20% acetic acid, and the dissolved material precipitated by the addition of 6 volumes of 95% ethanol. The precipitate was collected by centrifugation at 3000 R.P.M. for forty-five minutes. The residue was transferred to a disc of Whatman's #42 filter paper in a Gooch crucible and dried by solvent exchange, using 95% ethanol, acetone and ethyl ether, in that order. The final product (Code number, 4) was then air dried. The yield was 0.0425 gm.

METHOD III (Code number, 5):

Variation (a). As in Method I, umbilical cords of infants of blood group A were dried under acetone until used. After removal of the adhering blood, the cords were cut into small pieces and dried *in vacuo* overnight. The cords were then treated according to the method of Baer, Kabat and Knaub (7).

Concentrated hydrochloric acid was added to a suspension of

15.28 gm. of umbilical cord in 300 ml. of distilled water, until the pH was lowered to about 2.0, whereupon 6.2 mgm. of pepsin (Parke-Davis, 1:3000 N.F., crystalline) and 5.7 mgm. of magnesium sulfate were added. The pH was determined with "Accutint" pH Test Papers. The suspension was covered with toluene and incubated at 37°C. More concentrated hydrochloric acid was added daily to keep the pH at about 2.0. A second portion of pepsin and magnesium sulfate was added, and incubation continued as before. Similar to previous reports (7) enzymatic action, as judged by pH changes, became minimal after ten days.

The suspension was transferred to cellophane bags and dialysed for twenty-five hours against three changes of cold distilled water. The contents were readjusted to pH 2.0, 5.1 mgm. of pepsin and 5.3 mgm. of magnesium sulfate added, and the mixture incubated at 37°C. as before. Because the pH failed to rise during the third day of incubation, the digestion mixture was then filtered through fluted filter paper and the pH of the filtrate raised above 4.0 by the addition of solid sodium acetate. The filtrate was poured into 5 volumes of 95% ethanol, refrigerated for four to five hours, and the white gummy precipitate then collected in a Buchner funnel using Whatman's #42 filter paper. The precipitate was washed with 20 ml. of 95% ethanol and 10 ml. of ethyl ether, and dried <u>in vacuo</u> over P_2O_5. The yield of crude material was 1.0618 gm.

The dried material was suspended in 10 ml. of distilled water and left overnight at room temperature, after which it was centrifuged at 3000 R.P.M. for fifteen minutes. The supernatant liquid was decanted and preserved; the residue was extracted four times with 10 ml. of distilled water as before. Approximately 0.1 gm. of sodium acetate was dissolved in the combined supernatants which were then pipetted into 5

volumes of 95% ethanol, refrigerated, collected and dried as before. The yield at this stage was 0.8107 gm.

This material was deproteinized by the method of Morgan and King (62). The precipitate was shaken for twenty hours with 8 ml. of 90% (w/v) phenol, after which it was centrifuged at 2000 R.P.M. for three hours. The supernatant was decanted and preserved; the residue was extracted twice more in the same way, using 4 ml. of 90% (w/v) phenol. Only a trace of material was insoluble in the phenol. In order to obtain a 10% (v/v) ethanol concentration, 4.0 ml. of 1:1 90% (w/v) phenol-95% ethanol solution were mechanically stirred into the combined supernatant liquids and left overnight at room temperature, during which time a thin translucent gel was deposited from solution. This gel was collected by centrifugation at 1500 R.P.M. for twenty minutes, rubbed into suspension in 95% ethanol, filtered, and dried in vacuo over P_2O_5. The final product (Code number, 5-A) weighed 0.0291 gm.

Variation (b). A suspension of 22.71 gm. of umbilical cord, dried and milled as described above, was digested at pH 2.0 at 37°C. with 7.0 mgm. of pepsin in the presence of 6.8 mgm. of magnesium sulfate. The pH was adjusted daily. After five days 6.9 mgm. of pepsin and 6.5 mgm. of magnesium sulfate were added and incubation continued in the same way for another five days. The digestion mixture was filtered, and the group specific material in the filtrate precipitated by ethanol fractionation, water-extracted and reprecipitated as before. The yield of crude material was 5.1507 gm.

This precipitate was treated with one 50 ml. and four 20 ml. portions of 90% phenol according to the method of Morgan and King (62) cited above. The combined supernatants were mixed with the 1:1 phenol-

ethanol solution until an ethanol concentration of 10% (v/v) was reached. No precipitate formed on standing and so the ethanol concentration was increased in increments of 1%, two hours refrigeration being allowed between successive additions. When necessary the solution was left at room temperature overnight. At an ethanol concentration of 15% a thin gel was precipitated, collected by centrifugation, and suspended in 95% ethanol. The suspended material was filtered, and dried over P_2O_5 *in vacuo*. The final product (Code number, 5-B) weighed 1.1644 gm.

The phenol-insoluble residue was dissolved in 25 ml. of distilled water, mixed with 0.1 gm. of sodium acetate, and stirred into 5 volumes of 95% ethanol. The precipitate was collected in a Buchner funnel on Whatman's #42 filter paper and dried over P_2O_5 *in vacuo*. The final product (Code number, 5-B P.I.) weighed 3.8525 gm.

Variation (c). To test the merits of dessicating agents other than acetone, a group of umbilical cords of infants of blood group A was stored in 95% ethanol for ten days. The cords were cleaned of surface blood, cut into small pieces and placed in absolute ethanol for four days. Using 26.25 gm. of these dried umbilical cords, the method of Baer *et al.* (7) was followed, as described in the previous section. The initial alcohol precipitation yielded 6.9498 gm. of crude material. Ethanol fractionation of the water extracts precipitated 5.9021 gm. of material from solution. This was deproteinized by the method of Morgan and King (62) and precipitated from solution in 90% (w/v) phenol by the addition, with constant stirring, of 1:1 90% (w/v) phenol-95% ethanol solution to an ethanol concentration of 15% (v/v). A heavy precipitate formed which was collected by centrifugation, suspended in 95% ethanol, filtered, and dried over P_2O_5 *in vacuo*. The final product (Code number, 5-C) weighed 0.2732

gm.

Variation (d). A suspension of 180.2 gm. of umbilical cords of infants of blood group A in 2400 ml. of distilled water at pH 2.0, was incubated with 20.3 mgm. of pepsin for ten days at 37°C. according to the method of Baer et al. (7). The suspension was filtered, clarified by prolonged centrifugation, and the combined supernatant liquids evaporated under reduced pressure to a volume of about 450 ml. To this was added the 50 ml. of distilled water with which the evaporation flask was washed. This concentrate was centrifuged at 3000 R.P.M. for forty minutes. The supernatant solution was mixed with 0.1 gm. of sodium acetate and poured into 5 volumes of 95% ethanol. The resulting precipitate was collected by filtration and dried by solvent exchange. The precipitate (Code number, 5-E) weighed 4.8570 gm.

A comparison of the deproteinization methods of Morgan and King (62) and of Gerheim, Berkut and Gerheim (23) was made by treating portions of material 5-E by each method.

Following the method of Morgan and King (62), 2.0228 gm. of material 5-E were purified as described above. The agglutinogen was precipitated by the addition of 1:1 phenol-ethanol solution until a 10% (v/v) ethanol concentration was reached. The precipitate was collected by centrifugation, suspended in 95% ethanol, filtered, redissolved in 25 ml. of distilled water, and reprecipitated by ethanol fractionation. The final product (Code number, 5-E M-K) was dried first by solvent exchange and then air dried. The yield was 0.8781 gm.

To a 5% aqueous solution of 2.0042 gm. of material 5-E (volume, 40 ml.), were added 20 ml. of 10% sodium tungstate followed by the addition of 20 ml. of 0.6N sulfuric acid, after which the contents were shaken and

allowed to stand for fifteen minutes, as directed by Gerheim et al. (23). The solution was filtered and the filtrate dialysed in cellophane tubes against cold, running, tap water for about sixteen hours. The group specific material was obtained by pouring the dialysed solution into 10 volumes of acetone. The precipitate was collected by filtration, washed with large quantities of acetone and ethyl ether, and finally air dried. The yield of purified material (Code number, 5-E G) was 1.0352 gm.

The above procedure was repeated using 108.47 gm. of undried umbilical cords of infants of blood group O. The extracts were deproteinized by the Gerheim et al. procedure, dried by solvent exchange and then air dried. The final product (Code number, 5-o) weighed 1.1367 gm.

Variation (e). In the cord material used elsewhere in this research, there was always included a small amount of blood from inside the umbilical cord itself. Therefore, to ascertain separately the value of the umbilical cord and of this internal blood as a source of the agglutinogens, the following procedure was carried out.

Approximately ten undried, frozen, group A umbilical cords, weighing 185.8 gm. were ground up in a meat grinder, mixed with 200 ml. of distilled water and filtered, with suction, through a piece of clean cotton towelling in a Buchner funnel. This washing was repeated five times, the fifth and sixth wash waters being colorless. These wash waters were combined into two groups, the first group being composed of the first three extracts, the second group of the last three extracts. The washed umbilical cords were suspended in 750 ml. of distilled water. The three contents were adjusted to pH 2.0 with concentrated HCl, after which 3.0 mgm. of pepsin were added to each aqueous extract and 24.9 mgm. of pepsin to the cord suspension. As described above, these were then

incubated at 37°C. for ten days, precipitated from solution by ethanol fractionation and finally deproteinized by the method of Gerheim et al. (23), cited above. The deproteinized agglutinogen was precipitated in 10 volumes of acetone, filtered, washed with acetone and ether, and air dried. The initial ethanol fractionation precipitated 0.9207 gm. of material (Code number, 5-F-A) from the first group of wash waters, 0.5176 gm. of material (Code number, 5-F-B) from the second group of wash waters, and 9.3470 gm. of material (Code number, 5-F-W) from the umbilical cord suspension. The final purification yielded 0.6029 gm. of 5-F-A material, 0.3323 gm. of 5-F-B material, and 7.8403 gm. of 5-F-W material.

Variation (f). Umbilical cords of infants of blood group A, obtained and stored in a frozen condition, were thawed, washed free of adhering blood, ground up in a meat grinder, and suspended in 400 ml. of distilled water, the pH of which was then adjusted to about 2.0 with concentrated HCl. The ground up cords weighed 36.48 gm. After 4.8 mgm. of pepsin were added, the suspension was incubated at 37°C. Each day concentrated HCl was added to keep the pH close to 2.0. After five days this control was discontinued and the pH was allowed to rise naturally for five days, as the cord material autolysed, at 37°C. The digestion mixture was then filtered and the group specific material precipitated by ethanol fractionation. The yield of crude material was 0.9997 gm.

This precipitate was deproteinized by the method of Morgan and King (62). The ethanol-precipitated gel was collected by centrifugation, rubbed into suspension in 95% ethanol and refrigerated overnight. The suspended material was collected by filtration, dissolved in 25 ml. of distilled water and recovered by ethanol fractionation. The final product

(Code number, 5-8) was collected by filtration, dried first by solvent exchange and finally air dried. The yield was 0.6562 gm.

Variation (g). Umbilical cords of infants of blood group A were dried and milled as in Method I. A 700 ml. aqueous suspension of 38.65 gm. of umbilical cord was adjusted to pH 2.0 with concentrated HCl and, after the addition of 5.2 mgm. of pepsin and about 15 ml. of toluene, was incubated, with daily adjustment of the pH, at 37°C. After five days the suspension was adjusted to about pH 8.0 with sodium bicarbonate and 5.0 mgm. of pancreatin (Merck, U. S. P.) added. Incubation at 37°C. was continued for five days, sodium bicarbonate being added daily to keep the contents around pH 8.0.

Enzymatic digestion being complete, the suspension was centrifuged at 2500 R.P.M. for twenty-five minutes. The combined supernatant liquids were filtered, mixed with 0.2 gm. of sodium acetate and poured into 5 volumes of 95% ethanol. The precipitate (Code number, 5-PT) was collected by filtration and dried by solvent exchange. The yield was 11.76 gm. of light brown crystalline material.

This precipitate was extracted with six 100 ml. portions of distilled water on successive days. Each extract was clarified by centrifugation; the supernatant liquid was decanted and preserved. In order to investigate any diversity in the material dissolved during the various stages of extraction, these extracts were divided into three groups — group A, comprising the first two 100 ml. extracts; group B, comprising the third, fourth and fifth extracts; and group C, consisting of the final extract. Each of these groups was treated separately thereafter. The same method of purification was followed for all groups. This method was subdivided into three stages, as follows:

Stage 1 - About 0.1 gm. of sodium acetate was added to the aqueous extract which was then stirred into 5 volumes of 95% ethanol. The precipitate was collected by filtration and dried by solvent exchange. The group A suspension yielded 2.2806 gm. of precipitate (Code number, 5-PT-A), the group B suspension yielded 5.8311 gm. of material (Code number, 5-PT-B), while the group C precipitate (Code number, 5-PT-C) weighed 1.0307 gm.

Stage 2 - Each Stage 1 precipitate was shaken three times with twice its weight of distilled water for sixteen hours. After each extraction the suspension was clarified by centrifugation. The supernatants were combined and the group specific material precipitated, collected and dried as in Stage 1. The precipitate 5-PT-1A weighed 1.4323 gm., precipitate 5-PT-1B weighed 3.7106 gm., and precipitate 5-PT-1C weighed 0.9879 gm.

Stage 3 - Each of the three Stage 2 precipitates was shaken for sixteen hours on three consecutive days with 10 ml. of 90% (w/v) phenol per gm. of precipitate. Using a mechanical stirrer, 1:1 90% (w/v) phenol-95% ethanol solution was added to produce an ethanol concentration of 10% (v/v). On standing overnight, a thin gel was deposited and was collected by centrifugation. The gel was rubbed into suspension in 95% ethanol, refrigerated overnight, then dissolved in water and precipitated, collected and dried as in Stage 1. The yield of the purified 5-PT-A material was 1.1079 gm., that of 5-PT-B was 2.8282 gm., and that of 5-PT-C was 0.7723 gm.

Variation (h). A whole hog stomach, obtained fresh, and stored in a deep-freeze unit at -10 to -14°F., was rapidly thawed, and the

stomach lining, together with as much of the mucous material covering the lining as was possible to retain, was severed from the muscular wall. The oesophageal (non-glandular) region, as described by Grossman (72), was separated from the glandular region of the lining, and the two portions then cut into small pieces and dried separately in 95% ethanol for six days. The drying agent was then clarified by centrifugation, the residue thus obtained being mixed with the lining and immersed in absolute ethanol. After four days, the linings, being quite brittle, were dried in vacuo and ground in a Wiley mill. The dried non-glandular lining weighed 15.38 gm., the glandular lining 46.65 gm. To a 350 ml. aqueous suspension of glandular lining and a 125 ml. aqueous suspension of non-glandular lining, both adjusted to pH 2.0 with concentrated hydrochloric acid, were added 7.8 mgm. and 2.7 mgm. of pepsin, respectively. The suspensions were covered with toluene incubated at 37°C., the pH being adjusted daily. After five days, 7.9 mgm. of pepsin were added to the glandular suspension while 3.0 mgm. of pepsin were added to the non-glandular suspension. Enzymatic activity became minimal in two days in the glandular suspension, however measurable changes in pH continued until the fourth day in the non-glandular suspension. The digestion mixtures were filtered, the filtrate adjusted above pH 4.0 with sodium acetate, and the group specific material precipitated by ethanol fractionation as described above. In this way 11.77 gm. of precipitate were obtained from the glandular lining suspension, and 4.4 gm. of precipitate from the non-glandular suspension.

The glandular precipitate was extracted with one 50 ml. portion and two 30 ml. portions of distilled water, on successive days. After each extraction the solution was centrifuged at 2500 R.P.M. for forty minutes. The final residue was discarded. The combined supernatants

were mixed with 0.1 gm. of sodium acetate poured into 5 volumes of 95% ethanol, filtered, and dried over P_2O_5 in vacuo. The non-glandular precipitate was treated in the same way using half quantities of distilled water. This precipitation yielded 9.2820 gm. of material from the glandular suspension and 2.9011 gm. of non-glandular precipitate.

Final deproteinization was effected by the Morgan and King process (62) using 5.0028 gm. of glandular precipitate and 2.5103 gm. of non-glandular precipitate. The glandular precipitate was shaken for sixteen hours with 14 ml. of 90% (w/v) phenol. This shaking was repeated using separate 8 ml. portions of 90% (w/v) phenol on three consecutive days. The solution was clarified after each extraction by centrifuging at 2500 R.P.M. for one hour; the final phenol-insoluble residue was discarded. The group specific material which precipitated at a 10% (v/v) ethanol concentration was collected by centrifugation, suspended in ethanol, filtered, dried by solvent exchange and finally air dried. The non-glandular precipitate was extracted with one 7 ml. portion and three 4 ml. portions of 90% phenol as described above. The yield of purified material from the glandular lining (Code number, 5-HG) was 3.4779 gm., that from the non-glandular lining (Code number, 5-HN) was 1.0816 gm.

METHOD IV (Code number, 6): Umbilical cords of infants of blood group A were dried, washed and milled as in Method I. Proceeding as directed by Meyer, Smyth and Palmer (57), 20.05 gm. of umbilical cords were suspended in 500 ml. of distilled water and warmed on a steam bath at 55 - 60°C. The pH, which tended to drop slowly, was kept around 7.0 by the addition of small amounts of sodium carbonate. After approximately one hour, 10 gm. of sodium carbonate were added and the temperature of the suspension was

raised to 70°C. and maintained there for thirty minutes. The suspension was allowed to cool, was filtered, and the filtrate adjusted to about pH 5.0 with glacial acetic acid, after which it was refrigerated overnight. A fine precipitate settled out and was removed by centrifugation.

The supernatant solution was mixed with 150 ml. of 20% sulfuric acid to produce a 5% concentration of sulfuric acid, shaken with 100 gm. of kaolin for fifteen minutes and clarified by centrifugation. After the sediment was extracted once with 60 ml. of distilled water, the supernatant liquids were combined and poured into 2 volumes of 95% ethanol. The precipitate was filtered, rubbed into suspension in 95% ethanol, refiltered and dissolved in 100 ml. of distilled water. Acetic acid was added to a concentration of 2%, and the solution refrigerated overnight. After centrifugation at 2500 R.P.M. for fifteen minutes, the residue was washed with 5 ml. of distilled water which was added to the supernatants and poured into 2 volumes of 95% ethanol. The crude material was filtered and dried by solvent exchange. The yield was 2.3309 gm.

This precipitate was dissolved in 100 ml. of distilled water. A 25% basic lead acetate solution, made neutral to litmus with 2% acetic acid, was added until maximum cloudiness was reached. The precipitate was removed by centrifugation. The supernatant was adjusted to pH 9.0 with dilute ammonium hydroxide, after which 15 ml. of 25% basic lead acetate solution were added. The precipitate was removed by filtration, washed with about 10 ml. of water and suspended in 100 ml. of distilled water. The suspension was warmed to about 60°C. on a steam bath and CO_2 was passed through until there was no further precipitation of lead carbonate. The precipitate was removed by filtration. The filtrate was made acid to litmus with glacial acetic acid and precipitated by the addition of 3

volumes of 95% ethanol. The precipitate was collected by filtration, washed with 50 ml. of 95% ethanol, dissolved in 50 ml. of distilled water and precipitated by the addition of 600 ml. of glacial acetic acid. The precipitate was first dried by solvent exchange and finally air dried. The purified final product (Code number, 6) weighed 0.4025 gm.

METHOD V (Code number, 7):

Variation (a). The method of Bendich, Kabat and Bezer (10) was followed using 50.84 gm. of umbilical cords of infants of blood group A. A citrate-HCl buffer of approximately pH 2.3 was prepared according to directions for Sorenson's standard buffers (18). This was checked with a Beckman pH meter, model G, previously standardized with three buffer solutions prepared from Coleman Standard pH Tablets, and was found to be at pH 2.21. The cords, which had been dried and milled as in Method I, were suspended in 400 ml. of this buffer and 5.7 mgm. of pepsin were added. Toluene was poured on top and the suspension then incubated at 37°C. After five days the suspension was readjusted to about pH 2.3 with citrate-HCl buffer solution, 5.3 mgm. of pepsin were added and incubation resumed for another five-day period.

The digestion mixture was filtered in a Buchner funnel through Whatman's #42 filter paper; the filtrate was pipetted into 5 volumes of 95% ethanol and refrigerated overnight. The precipitate was collected by filtration, washed with 25 ml. of 50% ethanol, and dried over P_2O_5 in vacuo. The dried precipitate weighed 12.5022 gm.

Deproteinization of 6.3047 gm. of the crude material was effected by the method of Morgan and King (62). The combined phenol supernatants were clarified by centrifuging at 3000 R.P.M. for one hour, then

mechanically stirred with 32 ml. of 1:1 90% (w/v) phenol-95% ethanol, thus producing an ethanol concentration of 10% (v/v). On standing at room temperature overnight a thin translucent gel settled out, which was collected by centrifugation. It was suspended in 95% ethanol, filtered, dissolved in 25 ml. of distilled water and recovered by ethanol fractionation. This precipitate was collected by filtration, redissolved in 15 ml. of distilled water, and precipitated in 75 ml. of 95% ethanol. This precipitate was collected by filtration, washed with 10 ml. of 50% ethanol and dried over P_2O_5 *in vacuo*. The final product (Code number, 7-A) weighed 0.6031 gm.

Variation (b). A citrate-HCl buffer of pH 2.3 was prepared by Clark's directions (18). The pH was measured with a Beckman pH meter and was found to be 2.26. Umbilical cords of infants of blood group A, weighing 68.44 gm., were ground up in a meat grinder while still frozen, and suspended in 400 ml. of the buffer, to which 5.1 mgm. of pepsin were added. The suspension was covered with toluene and incubated at 37°C. After five days, the digestion mixture was centrifuged at 3100 R.P.M. for fifty minutes. The clear supernatant liquid was decanted and preserved. The residue was mixed with 40 ml. of the citrate-HCl buffer, in which 5.0 mgm. of pepsin were then dissolved, and once more covered with toluene. Incubation was resumed at 37°C. for five days, after which the contents were centrifuged at 3000 R.P.M. for thirty minutes. The supernatant solutions were combined, poured into 4 volumes of 95% ethanol and refrigerated overnight. The precipitate was concentrated by centrifuging at 2500 R.P.M. for twenty-five minutes, transferred to a Buchner funnel and collected on Whatman's #42 filter paper. This material was washed with 95% ethanol and acetone, and suspended in acetone overnight. The suspended

material was filtered, dissolved in 200 ml. of distilled water and recovered by ethanol fractionation. The precipitate was collected by filtration, dried first by solvent exchange and finally air dried. This material (Code number, 7-B) weighed 10.1124 gm.

It was observed that, during the drying by solvent exchange, a heavy curd-like precipitate formed in the filter flask, which then contained the ethanol and acetone used in the drying procedure, as well as the original ethanol solution, on addition of the ether. To investigate, this precipitate was collected by filtration and treated in an identical manner as the initial precipitate 7-B. It weighed 4.3655 gm. and was assigned the Code number 7-B-2.

Each precipitate was dissolved in about 100 ml. of distilled water and poured into 10 volumes of acetone. The precipitate was collected by filtration, washed with 75 ml. of ethyl ether, and air dried. The material was then deproteinized by the method of Morgan and King (62), and was collected and dried in the method cited above. The purified 7-B material (Code number, 7-B-1) weighed 2.3026 gm., the purified 7-B-2 material weighed 1.2007 gm.

METHOD VI (Code number, 8):

Variation (a). The autolysis of 50.86 gm. of ethanol-dried, coarsely divided, umbilical cords of infants of blood group A, was executed by the method of Bendich, Kabat and Bezer (10). The cords were suspended in 300 ml. of buffer solution at pH 2.0, prepared from Coleman Standard pH Tablets, covered with toluene and incubated at 37°C. for five days. The suspension was filtered through a sintered glass funnel. The filtrate was preserved; the residue was suspended in 200 ml. of the buffer

and incubated for another five days at 37°C. while further autolysis occurred. The autolysis mixture was filtered through Whatman's #40 filter paper. The residue was discarded. The filtrates were combined, evaporated under reduced pressure to about 200 ml., and poured into 5 volumes of 95% ethanol. The precipitate was collected by centrifugation, washed with 25 ml. of 50% ethanol, and dried over P_2O_5 in vacuo. The yield of crude material was 3.5798 gm.

A 1.5601 gm. portion of this crude material was deproteinized by the method of Sevag (71) using amounts of reagents proportional to those employed in Method I. The combined aqueous supernatants were clarified by centrifugation at 3000 R.P.M. for seventy minutes, after which they were poured into 3.5 volumes of 95% ethanol and refrigerated overnight. The precipitate was collected on Whatman's #42 filter paper in a Gooch crucible. The material was shaken with 2 ml. of distilled water overnight, and the undissolved material gathered by centrifuging at 3000 R.P.M. for forty-five minutes. The supernatant solution was preserved. The residue was re-extracted as before. The supernatants were combined, poured into 4 volumes of 95% ethanol and refrigerated overnight. The precipitate was collected by filtration as before, washed with 15 ml. of 50% ethanol, then with 25 ml. of 95% ethanol, and dried over P_2O_5 in vacuo. The yield of purified material (Code number, 8-A) was 0.4652 gm.

Variation (b). Following the autolysis procedure of Bendich, Kabat and Bezer (10), 38.42 gm. of umbilical cords of infants of blood group A, previously dried under ethanol and coarsely powdered in a Wiley mill, were suspended in 300 ml. of citrate-HCl buffer of about pH 2.3, prepared as directed by Clark (18). The pH was measured with a Beckman

pH meter and found to be 2.28. The suspension was covered with toluene and incubated at 37°C. while the umbilical cords autolysed. The autolysis was carried on for ten days after which the digestion mixture was centrifuged at 2500 R.P.M. for forty-five minutes. The residues were discarded; the supernatants were decanted into 4 volumes of 95% ethanol and refrigerated overnight. The precipitate was collected by filtration, washed with 50 ml. of acetone, and dried in vacuo overnight. The yield was 5.5808 gm.

This material was deproteinized by the method of Morgan and King (62). The combined phenol supernatants were cooled in the refrigerator and centrifuged at 3000 R.P.M. for four half-hour periods. The supernatants were maintained below 40°C. by cooling between periods of centrifuging. The group specific material was precipitated as a thin gel by the addition of 1:1 90% (w/v) phenol-95% ethanol to an ethanol concentration of 10% (v/v); a small crystal of sodium acetate was added to aid flocculation. The precipitate was collected by centrifugation, rubbed into suspension in 95% ethanol, and collected in a Gooch crucible on a disc of Whatman's #42 filter paper. The final product (Code number, 8-B) was dried over P_2O_5 in vacuo, and weighed 0.4683 gm.

Variation (c). A citrate-HCl buffer of about pH 2.3 was prepared, as directed by Clark (18), and the exact pH determined to be 2.31 with a Beckman pH meter. Using 400 ml. of this buffer, 140.82 gm. of undried umbilical cords of infants of blood group A were autolysed as in the previous method. The autolysis mixture was filtered through Whatman's #12 fluted filter paper. The filtrate was preserved. The residue was suspended in 100 ml. of the buffer and re-incubated for five days at 37°C., after which it was filtered and the residue incubated for five days, in

the same way. All three filtrates were kept separate and were treated in an identical manner.

Each filtrate was filtered three times through cotton batten in a Gooch crucible, then poured into 3 volumes of 95% ethanol. The precipitate was concentrated by centrifugation, transferred to a disc of Whatman's #42 filter paper in a Gooch crucible and dried by solvent exchange. A 5.6006 gm. portion of the precipitate from the initial autolysis filtrate was removed for assay, and was labeled as preparation 8-C-1. The remainder of the precipitate was extracted four times with approximately twice its weight of distilled water, by shaking for six hours, followed by centrifugation at 3000 R.P.M. for forty minutes. The supernatant solutions were combined and the dissolved material recovered by ethanol fractionation. The precipitate was collected by centrifugation, and suspended in acetone before being collected in a Gooch crucible as above. This material was deproteinized by the method of Morgan and King (62), and precipitated at an ethanol concentration of 10% (v/v). The material was suspended in 95% ethanol, filtered, dried by solvent exchange and finally air dried. The filtrate from the initial autolysis yielded 2.8990 gm. of final product (Code number, 8-C-1B), that from the second autolysis yielded 1.2363 gm. of purified material (Code number, 8-C-2), and the filtrate from the final autolysis yielded 0.6085 gm. of group specific substance (Code number, 8-C-3).

Variation (d). Autolysis of 38.42 gm. of umbilical cords of infants of blood group A, dried and milled as in Method I, was carried out in 300 ml. of citrate-HCl buffer at pH 2.18. The suspension was covered with toluene and incubated at 37°C. The contents were readjusted to about pH 2.3 after the fifth day, and five days later the autolysis

mixture was filtered twice in a Buchner funnel, first through Whatman's #40 and then through Whatman's #42 filter papers. The filtrate was centrifuged at 3000 R.P.M. for fifty minutes. The crude group specific substance was precipitated by ethanol fractionation, collected by filtration and dried over P_2O_5 *in vacuo*. The dried material weighed 2.2111 gm.

This precipitate was deproteinized by the Morgan and King (62) procedure. The purified material was deposited from solution in 90% (w/v) phenol by the addition of 1:1 90% (w/v) phenol-95% ethanol solution to a 10% (v/v) concentration of ethanol. The gel thus precipitated was collected by centrifugation and rubbed into suspension in acetone. The suspended material was collected and dried as in Method VI (b). The final product (Code number, 8-D) weighed 1.0217 gm.

The above procedure was repeated using 104.04 gm. of undried umbilical cords of infants of blood group B. The final product (Code number, 8-b) was dried by solvent exchange and then air dried. The yield was 0.1069 gm.

Variation (e). A whole hog stomach lining was dried and milled as in Method III (h), and subsequently autolysed at 37°C. in 350 ml. of a citrate-HCl buffer of pH 2.26. The dried lining weighed 29.84 gm. The suspension was covered with a layer of toluene. Ten days later the autolysis mixture was filtered twice through glass wool, and then through Whatman's #12 fluted filter paper. The filtrate was poured into 5 volumes of 95% ethanol and refrigerated overnight. The precipitate was collected by filtration, dried by solvent exchange and then air dried. The crude material weighed 3.1859 gm.

This material was deproteinized by the method of Morgan and King (62). The gel deposited by a 10% (v/v) concentration of ethanol was col-

lected by centrifugation, suspended in 95% ethanol and collected in a Gooch crucible on Whatman's #42 filter paper. The precipitate was shaken with separate 10 ml. portions of 90% (w/v) phenol for two sixteen-hour periods. When the phenol solutions had been combined and clarified by centrifugation, the group specific material was precipitated as before. This precipitate was collected, suspended and filtered as described above. The final product (Code number, 8-Hg) was dried by solvent exchange and then air dried. The yield was 1.8112 gm.

The above procedure was repeated using a complete, undried hog stomach lining weighing 56.06 gm. The initial ethanol precipitate weighed 4.3986 gm. The final product (Code number, 8-Hg-B) weighed 2.4435 gm.

Variation (f). An entire undried hog stomach lining weighing 60.47 gm. was cut up and suspended in 300 ml. of McIlvaine's standard phosphate-citric acid buffer at about pH 2.3 prepared as directed by Clark (18). The suspension was found to be at pH 2.22, using a Beckman pH meter. The contents were covered with toluene and incubated at 37°C. for ten days. The autolysis mixture was filtered through glass wool, then Whatman's #12 fluted filter paper, and the filtrate clarified by centrifugation. The supernatant solution was poured into 5 volumes of 95% ethanol. The resulting precipitate (dried weight, 3.3167 gm.) was treated as in the previous method. The final product (Code number, 8-Hg-A) weighed 2.0262 gm.

METHOD VII (Code number, 9): Proceeding according to the directions of Bray, Henry and Stacey (14), 68.08 gm. of umbilical cords of infants of blood group B, dried and milled as in Method I, were suspended in 300 ml.

of distilled water and heated on a hot plate for twenty-five minutes at about 95°C. The suspension had been adjusted to pH 7.0 with calcium carbonate; after fifteen minutes, more was added, to keep the pH constant. The contents were allowed to cool to room temperature after which it was filtered through Whatman's #4 filter paper in a Buchner funnel. The viscous filtrate was deproteinized by shaking for twenty minutes with 20 ml. of chloroform and 2 ml. of amyl alcohol, and centrifuging at 2400 R.P.M. for eighty minutes. The material separated into three well-defined layers (cf. Method I). The large top aqueous layer was separated from the middle layer of coagulated protein by decantation and was deproteinized as before another five times. Thereafter, no further protein coagulation was observed. The final supernatants were adjusted to about pH 4.2, as determined with "Accutint" pH Test Papers, with sodium acetate, and poured into 3.5 volumes of 95% ethanol. The solution was refrigerated overnight, filtered, and the precipitate dried by solvent exchange.

This material was dissolved in 50 ml. of distilled water, to which five drops of 5% calcium chloride had been added, and poured into 5 volumes of 95% ethanol. The precipitate was concentrated by centrifugation at 3250 R.P.M. for fifteen minutes, transferred to a disc of Whatman's #42 filter paper in a Gooch crucible and there dried by solvent exchange. The precipitate was dissolved in 35 ml. of distilled water, filtered, and poured into 5 volumes of 95% ethanol. This material was collected in a Gooch crucible as before, dried by solvent exchange, and finally air dried. The final product (Code number, 9-b) weighed 0.0908 gm.

METHOD VIII (Code number, 10): The extraction method of Sevag (71) was employed in the treatment of 31.51 gm. of umbilical cords of infants of

blood group A, which had been dried and milled as in Method I. The cords were mixed in a mortar with 50 ml. of distilled water, and frozen in a deep freeze unit at -10 to -14°F., after which they were alternately thawed and frozen six times. The thawed material was mixed with 500 ml. of distilled water, 200 ml. of chloroform and 20 ml. of amyl alcohol, and shaken for sixteen hours. The mixture was centrifuged at 2400 R.P.M. for one hour. The aqueous supernatants were decanted, combined and preserved. The two layers beneath were mixed with 500 ml. of distilled water, 50 ml. of chloroform and 10 ml. of amyl alcohol, then shaken and centrifuged as before.

This process was repeated once more, after which all the supernatants were combined and evaporated to dryness under reduced pressure. The solution was warmed to 25°C. before evacuation commenced but no attempt was made to keep the solution at constant temperature thereafter. The residue was dissolved in 250 ml. of distilled water; the solution was clarified by centrifugation at 2500 R.P.M. for thirty-five minutes. The supernatant solutions were deproteinized once again, as before. The mixture was centrifuged at 3000 R.P.M. for forty minutes, after which the aqueous supernatants were drawn off with a pipette and evaporated to dryness as before.

The residual material was dissolved in 150 ml. of distilled water, filtered and poured into 5 volumes of 95% ethanol. The precipitate was collected by filtration, dried by solvent exchange, and finally air dried. The final product (Code number, 10) weighed 1.2362 gm.

CHARACTERIZATION OF THE EXTRACTED MATERIALS

The products isolated by the preceding methods were character-

ized by assaying the following properties:

(a) Blood group specific activity was determined at the Rh laboratory of the Winnipeg Children's Hospital by the isoagglutination inhibition technique of Morgan and King (62).

(b) Optical activity was determined with a polarimeter using sodium light. The solutions were placed in a 1 decimeter tube with an inner volume of 5.00 ml. All solutions were at 31.8°C. when used. Five readings were made for each sample. These were averaged and the mean reading used in calculating the optical rotation. The error was estimated to be 2 degrees.

(c) Total nitrogen content was determined by micro-Kjeldahl (cf. 26, 30, 43). The group specific material was dissolved in distilled water in concentrations calculated to contain between 1.0 and 1.5 mgm. of nitrogen per ml., assuming a nitrogen concentration of 5%. One ml. of sample was digested in a 100 ml. Kjeldahl flask with 1 ml. of concentrated sulfuric acid, 0.5 gm. of potassium sulfate and 1 ml. of 5% mercuric sulfate in 7 N sulfuric acid, for thirty minutes after the mixture cleared.

The distillation was carried out as directed by Hiller, Plazen and Van Slyke (30), except that solid NaOH, and the Hengar air condenser apparatus, were used. As was suggested (30), the 0.1 gm. of powdered zinc was added to the digested material before the NaOH. The ammonia was distilled into 10 ml. of approximately 0.01N HCl prepared by the directions of Kabat and Mayer (43), containing 1 ml. of cresol red indicator. The distillate was made alkaline with approximately 0.01N KOH and back titrated with the standard HCl.

The KOH was standardized by titration with potassium acid

phthalate; the HCl was then standardized by titration with the KOH. The acid was standardized against the KOH daily while the nitrogen determinations were carried out.

Each determination was done in triplicate. A blank was run with every six samples, and a known standard was assayed with every twelve samples. The error was estimated to be not greater than 3%.

RESULTS AND DISCUSSION

Results:

This study of the umbilical cord as a source of the blood group A substance showed conclusively that an A agglutinogen of great potency could be extracted in reasonable yield from this material.

The results of the assays for blood group A and B specific activity have been detailed in Tables I and II. The total nitrogen content, uncorrected for ash or moisture, and the optical activity, where clear, colorless solutions were obtained, have been listed, along with a summary of the blood group specific activities, in Tables III and IV.

Discussion of the Results:

The blood group specific activities of the agglutinogens obtained from umbilical cords of infants of blood group A showed an extremely wide variation as shown in Table I.

The isoagglutination inhibition test is a method of demonstrating the presence of agglutinogens by an absorption reaction. The material under investigation was dissolved in serial dilutions and incubated with known dilutions of agglutinin for a suitable time -- usually about sixty minutes. When material from A cords or from hog stomach linings was tested then B serum was used as a source of agglutinin; conversely, when material from group B cords was tested then A serum was used. After incubation, washed erythrocytes of the same group as the source material were added, shaken thoroughly, and allowed to react for some time.

Tables I and II show the maximum dilutions of the investigated material which inhibited agglutination of the erythrocytes, both partially and completely, in the various dilutions of agglutinins. Where no inhibition occurred, the activity of the preparation was listed as "nil".

TABLE I.

BLOOD GROUP SPECIFIC ACTIVITY OF THE MATERIALS FROM GROUP A UMBILICAL CORDS

CELLS		GROUP A_1						GROUP $A_2 > A_1$					
SERUM DILUTION		1/4		1/16		1/64		1/4		1/16		1/64	
Preparation	Concentration in gm. / ml.	C *	P #	C *	P #	C *	P #	C *	P #	C *	P #	C *	P #
3	0.05	nil	nil	nil	nil	nil	nil						
4	0.1 x	nil	nil	nil	nil	nil	nil						
5-A	0.8	nil	nil	nil	10^{-1}	10^{-1}	10^{-3}						
5-B	0.1	nil	10^{-1}	nil	10^{-3}	10^{-1}	10^{-4}						
5-B P.I.	0.20	nil	nil	nil	10^{-1}	nil	10^{-1}	nil	10^{-2}	10^{-2}	10^{-5}	10^{-4}	10^{-5}
5-C	0.01	nil	nil	nil	10^{-1}	10^{-1}	10^{-3}						
5-E	0.1	nil	10^{-1}	nil	10^{-3}	nil	10^{-5}	10^{-2}	10^{-4}	10^{-3}	10^{-4}	10^{-7}	
5-E G	0.1	nil	10^{-1}	nil	10^{-3}	10^{-1}	10^{-4}	10^{-2}	10^{-3}	10^{-2}	10^{-4}	10^{-6}	
5-E M-K	1% (w/v)	nil	nil	nil	10^{-4}	10^{-4}	10^{-5}						
5-F-A (pure)	0.1	nil	10^{-1}	nil	10^{-2}	10^{-2}	10^{-4}						
5-F-B (pure)	0.1	10^{-1}	10^{-3}	10^{-1}	10^{-4}	10^{-4}	10^{-5}						
5-F-W (crude)	0.1	nil	10^{-1}	10^{-1}	10^{-2}		10^{-3}	10^{-1}	10^{-4}	10^{-2}	10^{-3}	10^{-3}	
5-F-W (pure)	0.1	nil	nil	nil	10^{-2}	nil	10^{-3}	nil	10^{-2}	10^{-2}	10^{-3}	10^{-4}	
5-8 (crude)	0.1	nil	nil	nil	10^{-1}	10^{-1}	10^{-2}						
5-8 (pure)	1% (w/v)	nil	10^{-1}	nil	10^{-1}	10^{-1}	10^{-2}						
5-PT-A (Stage 1)	0.1	nil	10^{-1}	10^{-1}	10^{-2}	10^{-1}	10^{-3}						
5-PT-B (Stage 1)	0.1	nil	nil	nil	nil	nil	10^{-1}						
5-PT-C (Stage 1)	0.1	nil	nil	nil	10^{-1}	nil	10^{-1}						
5-PT-A (Stage 2)	0.1	nil	10^{-2}	10^{-1}	10^{-3}	10^{-2}	10^{-3}						
5-PT-A (Stage 3)	0.1	10^{-1}	10^{-3}	10^{-2}	10^{-4}	10^{-4}	10^{-7}						
5-PT-B (Stage 3)	0.1	nil	nil	nil	10^{-2}	nil	10^{-4}						
5-PT-C (Stage 3)	0.1	nil	nil	nil	10^{-1}	nil	10^{-3}						
6 (crude)	0.1	nil	10^{-1}	nil	10^{-3}	10^{-1}	10^{-3}						
6 (pure)	0.1	nil	10^{-1}	nil	10^{-2}	10^{-1}	10^{-3}						
7-A (crude)	0.1 x	nil	nil	nil	nil	nil	10^{-3}						
7-A (pure)	0.1	nil	10^{-1}	nil	10^{-2}	10^{-3}	10^{-4}						
7-B	0.1 x	nil	nil	nil	10^{-1}	nil	10^{-4}						
7-B-1	0.1	nil	10^{-1}	10^{-1}	10^{-2}	10^{-3}							
7-B-2	0.1	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-2}	10^{-3}						
8-A (crude)	0.1 x	nil	10^{-1}	10^{-1}	10^{-2}	10^{-1}	10^{-4}						
8-A (pure)	0.1	nil	10^{-1}	nil	10^{-2}	10^{-2}	10^{-5}						
8-B (pure)	0.1	10^{-2}	10^{-2}	10^{-2}	10^{-4}	10^{-4}	10^{-5}						
8-C-1	0.1	nil	10^{-1}	10^{-1}[A]	10^{-3}[B]	10^{-1}[C]	10^{-3}[D]	10^{-2}	10^{-3}				
8-C-1B (crude)	0.1	nil	10^{-3}	10^{-3}[B]	10^{-7}	10^{-7}	10^{-8}‡						
8-C-1B (pure)	0.1	nil	10^{-4}	10^{-4}	10^{-8}‡	10^{-8}‡	10^{-8}‡						
8-C-2	0.1	nil	10^{-1}	nil[C]	10^{-2}	10^{-1}[C]	10^{-3}[B]	10^{-1}	10^{-2}				
8-C-3	0.1	10^{-2}	10^{-4}	10^{-8}‡	10^{-8}‡	10^{-8}‡	10^{-8}‡						
8-D (pure)	0.2 x	10^{-1}	10^{-3}	10^{-2}	10^{-6}	10^{-4}	10^{-7}‡						
10	0.1	nil	10^{-1}	nil	10^{-3}	10^{-1}	10^{-3}						

* Complete inhibition of isoagglutination to and in this dilution.

\# Partial inhibition of isoagglutination to and in this dilution.

x Approximate concentration; solution not complete.

‡ No further dilutions tested.

[A] True dilution 1/10.

[B] True dilution 1/1250.

[C] True dilution 1/50.

[D] True dilution 1/6250.

TABLE II.

BLOOD GROUP SPECIFIC ACTIVITY OF THE MATERIALS FROM GROUP B CORDS AND FROM HOG STOMACHS

CELLS		GROUP A_1						GROUP B					
SERUM DILUTION		1/4		1/16		1/64		1/4		1/16		1/64	
Preparation	Concentration in gm. / ml.	C *	P #	C *	P #	C *	P #	C *	P #	C *	P #	C *	P #
Group A Specific Materials from Hog Stomachs													
5-HG (3rd ethanol ppt.)	0.1	10^{-3}	10^{-4}	10^{-4}	10^{-6}	10^{-5}	10^{-7}‡						
5-HG (pure)	0.1	10^{-4}	10^{-5}	10^{-5}	10^{-7}	10^{-6}	10^{-7}‡						
5-HN (crude)	0.1	10^{-1}	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-5}						
5-HN (pure)	0.09	10^{-3}	10^{-4}	10^{-3}	10^{-7}‡	10^{-5}	10^{-7}‡						
8-Hg (crude)	0.1	10^{-2} ≠	10^{-3} ≠	10^{-3} ≠	10^{-4} ≠								
8-Hg (pure)	0.09	nil	10^{-1}	10^{-1}	10^{-3}	10^{-3}	10^{-7}‡						
8-Hg-A (crude)		nil	nil	nil	10^{-1}	nil	10^{-1}						
8-Hg-A (pure)		10^{-1} ≠	10^{-3} ≠	10^{-3} ≠	10^{-5} ≠								
8-Hg-B (2nd ethanol ppt.)	0.07	10^{-3}	10^{-6}	10^{-5}	10^{-7}‡	10^{-6}	10^{-7}‡						
8-Hg-B (pure)	0.05	10^{-2}	10^{-7}‡	10^{-7}‡		10^{-7}‡							
Group B Specific Materials from Group B Cords													
8-b (crude)	0.1							ril	nil	nil	10^{-1}	nil	10^{-2}
8-b (pure)	0.1							nil	nil	nil	nil	nil	10^{-2}
9-b (pure)	0.01							nil	10^{-1}	nil	10^{-2}	nil	10^{-3}

* Complete inhibition of isoagglutination to and in this dilution.

\# Partial inhibition of isoagglutination to and in this dilution.

‡ No further dilutions tested.

≠ $A_2 > A_1$ cells used.

TABLE III.

PROPERTIES OF SUBSTANCES OBTAINED FROM GROUP A CORDS

PREPARATION	YIELD	TOTAL NITROGEN	$(\alpha)_D$	ACTIVITY			
				A_1 Cells		A_2 Cells	
				Dilution of B Serum		Dilution of B Serum	
				1/50	1/64	1/16	1/64
	per cent	per cent	degrees	mgm. A specific material producing complete inhibition.			
3	0.6	7.4	—		nil		nil
4	0.2	6.6	—		nil		nil
5-A	0.2	5.9	+21		80		
5-B	5.1	5.5	—		10		
5-B P.I.	16.9	4.8	+ 7		nil	2	2×10^{-2}
5-C	1.0	5.3	+16		1		
5-E	2.7	5.9	+22		nil	10^{-1}	10^{-2}
5-E G	51.7 *	5.2	+14		10	1	10^{-2}
5-E M-K	43.4 *	5.4	+14		10^{-3}		
5-F-A (crude)		5.9	—				
5-F-B (crude)		6.1	- 5				
5-F-W (crude)	5.0	6.1	+24			1	10^{-1}
5-F-A (pure)		5.6	—		1		
5-F-B (pure)		5.5	+ 2		10^{-2}		
5-F-W (pure)	4.2	5.4	+19		nil	1	10^{-2}
5-8 (crude)	2.7	6.3	+24		10		
5-8 (pure)	1.8	5.9	+20		10		
5-PT	30.4	6.4	—				
5-PT-A (Stage 1)	5.9	5.7	+22		10		
5-PT-B (Stage 1)	15.1	6.8	+26		nil		
5-PT-C (Stage 1)	2.7	6.3	+23		nil		
5-PT-A (Stage 2)	3.7	5.6	+22		1		
5-PT-A (Stage 3)	2.9	5.1	+19		10^{-2}		
5-PT-B (Stage 3)	7.3	6.2	+21		nil		
5-PT-C (Stage 3)	2.0	5.8	+18		nil		
6 (crude)	11.6	6.2	—		10		
6 (pure)	2.0	5.9	+19		10		
7-A (crude)	24.6	6.0	+24		nil		
7-A (pure)	1.2	5.6	+18		10^{-1}		
7-B	14.8	6.1	+19		nil		
7-B-1	3.4	5.3	+15		10^{-1}		
7-B-2	1.7	5.1	+12		1		
8-A (crude)	7.0	7.0	—		10		
8-A (pure)	0.9	5.7	+21		1		
8-B (crude)	14.5	6.3	—				
8-B (pure)	1.2	5.6	+15		10^{-2}		
8-C-1	4.0	6.1	+23	10			
8-C-1B (crude)	2.6	5.6	+18		10^{-5}		
8-C-1B (pure)	2.1	5.5	+18		10^{-6} #		
8-C-2	0.9	6.0	+20	10			
8-C-3	0.4	5.3	+16		10^{-6} #		
8-D (crude)	5.8	5.8	—				
8-D (pure)	2.7	5.5	+11		10^{-2}		
10	3.9	5.8	—		10		

* Yield calculated from original weight of the individual portions of crude material which were purified.

No further dilutions tested.

T A B L E I V.

PROPERTIES OF PREPARATIONS FROM HOG STOMACH LININGS, FROM GROUP B, AND FROM GROUP O UMBILICAL CORDS.

PREPARATION	YIELD	TOTAL NITROGEN	$(\alpha)_D$	ACTIVITY			
				Dilution of B Serum		Dilution of A Serum	
				1/16	1/64	1/16	1/64
	per cent	per cent	degrees	mgm. group specific material producing complete inhibition.			
Materials from Hog Stomachs							
5-HG (3rd ethanol ppt.)	19.9	6.6	-16	10^{-2}	10^{-3}		
5-HG (pure)	69.5 #	6.1	-21	10^{-3}	10^{-4}		
5-HN (crude)	18.9	6.3	-15	1	10^{-1}		
5-HN (pure)	42.1 #	6.1	-20	10^{-1}	10^{-3}		
8-Hg (crude)	10.7	7.4	-27	10^{-1}			
8-Hg (pure)	6.1	6.4	-18	10	10^{-1}		
8-Hg-A (crude)	5.5	6.1	-20	nil	nil		
8-Hg-A (pure)	3.3	5.6	-13	1 *			
8-Hg-B (2nd ethanol ppt.)	7.8	6.7	-23	10^{-3}	10^{-4}		
8-Hg-B (pure)	4.4	6.2	-15	10^{-5}	10^{-5}		
Materials from Group B Cords							
8-b (crude)		4.8	+ 6			nil	nil
8-b (pure)	0.1	4.4	+11			nil	nil
9-b (pure)	0.1	4.7	+ 8			nil	nil
Materials from Group O Cords							
5-O	1.1	5.8	-20				

* Concentration unknown (cf. Table II.)

\# Yield calculated from weight of the individual portions of crude material which were purified.

The concentration of the initial solution of agglutinogen was listed so that the amount of agglutinogen causing complete or partial inhibition of agglutination at any serum dilution could be calculated. These quantities, in mgm., are given in the summaries of the activities, in Tables III and IV.

It will have been noted that certain of the preparations were tested with more than one type of erythrocytes, designated as A_1 and $A_2 > A_1$ cells. The existence of more than one type of A erythrocytes — that is, of sub-groups — resulted from the agglutinogen A not being a single substance but one which included two main sorts of properties designated as A_1 and A_2. There are at least two types of blood group A, and perhaps more (cf. 32, 77). Blood of type A_1 absorbs the agglutinin anti-A more strongly than does type A_2 blood. Consequently the serum of most group B individuals contains at least two varieties of anti-A: one of these, designated as anti-A_1, agglutinates blood containing agglutinogen A_1, but reacts hardly at all with A_2 blood; the other, designated as anti-A, reacts almost equally on bloods of both sub-groups (11, 77).

When erythrocytes are agglutinated by a group specific serum they are coated agglutinins — that is, the erythrocytes "bind" the agglutinin. However if sufficient agglutinogen is added to the agglutinin before erythrocytes are added, the absorption of the agglutinin will occur and the agglutinogen will bind the agglutinin. This results in there being no free agglutinin available for absorption by the erythrocytes and hence no agglutination occurs.

It must be noted that the cells designated as $A_2 > A_1$ were not true A_2 cells but instead were cells, the reaction of which, was closer to that of A_2 cells than A_1 cells.

A preparation was considered to be inactive if, in its most highly purified form, it failed to cause complete inhibition of isoagglutination in dilutions of not less than 1:10,000 in a serum dilution of 1:64.

The results (cf. Table I) showed that in all cases tested the material under investigation was equally, or more, active when tested with $A_2 > A_1$ cells than with A_1 cells. For example, preparation 5-B P.I. showed almost no ability to inhibit the isoagglutination of A_1 cells while the agglutination of the pseudo-A_2 cells in serum diluted 1:64 was completely inhibited by a dilution of 1:10,000 of the same preparation. Similarly, preparation 5-E in serum diluted 1:64 gave partial inhibition of A_1 cells at a dilution of 1:100,000, but of pseudo-A_2 cells a dilution of 1:10 million. This was in agreement with the literature (77) which stated that the relative sensitivity of the A agglutinogen in the four sub-groups could be indicated as:

$$A_1 > A_1 B > A_2 > A_2 B.$$

In general, the activities of the materials obtained from hog stomach linings were not as active as those obtained by other investigators (57), when judged by the minimal amounts required for complete inhibition of isoagglutination. Of the five preparations from the hog stomach linings only one, 8-Hg, failed to meet the criterion and could be considered inactive. Two preparations, 5-HG and 8-Hg-B, compared very favorably with other preparations reported (9, 38, 57) from this source. The purified 8-Hg-B preparation inhibited isoagglutination completely at a dilution of 1:10 million. Only the preparation of Meyer et al. (57) exceeded this activity; their preparation was detectable serologically in amounts of 5×10^{-10} gm.

A great many of the preparations obtained from group A umbilical

cords fell short of the criterion of activity, and some preparations, such as 3 and 4, showed no activity whatsoever. Of approximately twenty-six preparations, seven were active towards anti-A_1 and three others were active towards anti-A. These active preparations compared favorably with any such preparation reported in the literature. Preparations 8-C-1B and 8-C-3 exceeded the activities of the agglutinogens isolated from such sources as human saliva (6, 53), ovarian cyst fluid (6, 61), human blood (6, 74), pepsin (14), urine (20), hog gastric mucosa (57), meconium (68) and hog gastric mucin (84), but were less active than preparations from horse stomach lining (7), horse saliva (48) and peptone (51).

The preparations from group B umbilical cords showed little or no ability to inhibit isoagglutination. None was able to produce complete inhibition; the most potent preparation, at a relative dilution of 1:10,000, partially inhibited isoagglutination in serum diluted 1:64. In general, the B agglutinogens isolated from a given source have been found by many workers (7, 8, 21, 61, 68) to be less active than the A agglutinogens from the same source. Morgan and van Heyningen (61) had great success in extracting an A group specific substance from pseudomucinous ovarian cyst fluids, but never encountered "a really potent" B fluid in their research. The least active B substance reported, 0.25 mgm. showed group B activity, was extracted from meconium by Rapoport and Buchanan (68). The most active B substance was prepared by Witebsky and Klendshoj (79) from human saliva and gastric juice. In dilutions of 1:1 million these preparations inhibited isoagglutination completely. The most active group B preparation from umbilical cords, 9-b, was slightly more active than the meconium B substance (68), 0.1 mgm. being detectable serologically.

The nitrogen content has been taken as the measure of purity:

the lower the amount of nitrogen the more nearly pure the preparation. While this permitted an accurate and readily obtainable standard of comparison, other properties did not always corroborate this assumption. The percentages of nitrogen reported in Tables III and IV were calculated from the weight of dried A, B or O substance used in the micro-Kjeldahl determination; neither the ash nor the moisture content of the preparations was determined. As a result the nitrogen values reported were slightly lower than they would have been if calculated on an ash free basis.

That nitrogen content actually is a criterion for purity has been illustrated by the marked correlation between the activity of a preparation and the amount of nitrogen it contained. In all but three cases, an increase in activity was accompanied by a decrease in nitrogen content resulting from purification. The correlation held only among the various stages of purification of a given preparation and while there appeared to be no inter-relation of the nitrogen contents of different preparations and their corresponding activities, there existed a characteristic nitrogen value for the preparations from each of the blood groups. The nitrogen content of B substances proved to be significantly less than that of the A substances.

The most active preparations obtained in this research contained considerable amounts of nitrogen. This was in agreement with the findings of Aminoff *et al.* (6) who reported 5.7% nitrogen, Kabat *et al.* (37) who reported 5.3 - 6.0% nitrogen, and Rapoport and Buchanan (68) who reported 5.1 - 7.1% nitrogen, in their most active purified preparations. The substances obtained from group A umbilical cords fell readily within this range of nitrogen content when purified. Indeed, except for preparation 3, even the crude preparations did not exceed a nitrogen content of 7.1%.

These results suggested that the limits of purification, obtainable by the methods of extraction and deproteinization used, had been reached and that the nitrogen present was not that of protein impurities, but rather was that of the amine groups of the hexosamines. The diversities observed in the percentage of nitrogen found in different preparations could have been due to variations in the proportions of carbohydrate moiety present, although since no spectrophotometric analysis was made, the absence of nucleic acid impurities (34) could not be established.

Examination of the assays of activity showed (cf. Table II) that while the A preparations were, in some cases, very potent, such was not the case with the preparations from group B umbilical cords. While the B substances obtained from meconium (68) contained about 6% nitrogen, Witebsky and Klendshoj (79) reported a human gastric juice B substance containing 1.5 - 1.6% nitrogen on an ash-free basis. More recent preparations by these same investigators (80) yielded nitrogen values in the same range as well as values between 3 and 4% although the activities were comparable. This would indicate that either the group B substance is not present in umbilical cord or that the purification of the umbilical cord B substance was unsuccessful, resulting in the great difference in activities.

Although it is of little or no significance, there being only one preparation from group O cords, Morgan and Waddell (63), using a variation of the method of Morgan and King (62), obtained from ovarian cyst fluid an O substance of moderate activity, containing 5.7% nitrogen. These results were almost identical with the nitrogen determinations carried out on the umbilical cord O substance.

The optical activity was found to be highly characteristic of

the source material. In the determinations of the optical activity large errors were incorporated due to the necessarily high dilutions of the solutions tested, which resulted in a small observed rotation. The specific rotation $(\alpha)_D$, using sodium light, was calculated from the equation,

$$(\alpha)_D = \frac{a}{w \times l}$$

where: a was the observed rotation,

w was the weight in grams of the preparation dissolved in 1 ml. of the observed solution, and

l was the length of the tube in decimeters.

The results recorded in Table III showed that very little difference existed in the preparations with respect to optical activity. Once again, for a given preparation, there was a marked correlation between nitrogen content and specific rotation, the latter decreasing with decreasing nitrogen content. All preparations from group A and group B cords, with the exception of 5-F-B, were dextrorotatory. Similarly, other workers (6, 7) encountered isolated preparations with optical rotations contrary to those usually found in preparations from that particular source. For example, Aminoff et al. (6) isolated a laevorotatory material with an optical rotation approximating that of crude 5-F-B preparation. This substance too reversed both the normal direction of optical rotation and the correlation between nitrogen content and specific rotation. As yet, no explanation has been given by these other workers. Other intra-special variations have been observed (38, 41). The preparation from group O cords was laevorotatory and had a specific rotation which agreed closely with the findings of Morgan and Waddell (63). The abnormally low optical activity of the phenol-insoluble preparation 5-B P.I., however, was

opposed to the findings of Aminoff et al. (6) who reported that their phenol-insoluble preparation had an abnormally large specific rotation. The preparations from hog stomach linings, being laevorotatory, were in agreement with the reports of other investigators (6, 15).

Considering now the various methods of extraction it will have been noted that in each there were four general steps — the preparation of the umbilical cords; the extraction of the agglutinogen; the purification of the extracted material, and, finally, the drying of the preparation.

The procedure of pretreatment of the umbilical cords by drying in acetone, suggested by Meyer and Palmer (56), was used at first because it provided a simple method of preserving and handling the cords. The method produced a material sufficiently dry to permit the use of a Wiley mill and hence a high degree of comminution was obtainable. No refrigeration was required; the dried cord material could be stored at room temperature for more than a year without deterioration of either the cord itself or the agglutinogen.

Ethanol was found to be much superior to acetone as a dessicant. The latter tended to "fix" the blood on the cords, making its removal difficult; the alcohol did this to a much less extent. As well, the alcohol was faster by several days in effecting adequate dessication of the cords, although cutting the cords into small pieces doubtlessly affected the rate of dessication.

To prevent waste, the solvent was distilled from the extracted moisture and the suspended blood. The dessicants had to be removed from the cords by warming in a forced-ventilation incubator at 37°C., or, as in variations (a), (b) and (c) of Method III, by continuous overnight evacua-

tion by a high-vacuum pump. The latter procedure was found to be less efficient than heat-drying, but physical deterioration of the umbilical cords was much less pronounced. The vacuum-drying method caused serious dilution of the oil in the vacuum pump. The ethanol, to be most effective, had to be anhydrous, thus making the production of absolute ethanol essential. These necessary extra steps detracted from the advantages of this method of dessication.

However, dessication was found to be both unnecessary and inferior to refrigeration. The umbilical cords were packed in pairs in small boxes of thin cardboard, and frozen solid in the freezing compartment of a refrigerator. So packed, they could be transported for several miles without refrigeration without showing signs of thawing. While in the laboratory, the cords were stored in a deep-freeze unit at -10 to -14°F. When the cords were required, the adhering blood was removed by holding the cords in a stream of cold water and rubbing gently with a rubber policeman. It was found to be more convenient and entirely feasible to re-freeze the cords and grind them in a meat grinder while still unthawed. This method of preservation and handling eliminated the possibility of denaturation of any thermo-labile substance in the cords. Umbilical cords kept for as long as eleven months in the frozen condition showed no deterioration and gave good yields when used in Method V.

The advantages possessed by the freezing method were so numerous as to exclude entirely the use of dessicants in pretreating or preserving the umbilical cords. The one requirement for this method was adequate and continuous refrigeration such as is obtainable with an ordinary refrigerator. The cord material was not sub-divided to as great an extent as when dried cords were milled, but the yields of both procedures were comparable,

and the activities of the preparations obtained from the undried cords were greatly superior.

The preparation obtained by the procedure detailed in Method I was neither active nor highly purified. Deproteinization, as in Method VIII, was by Sevag's method (71). However, in Method I, failure to produce rupturing of the cell walls by alternate thawing and freezing resulted in preparation 3 having the highest nitrogen content. The alternate thawing and freezing carried out in Method VIII resulted in a 600% increase in yield and the extraction of a much more nearly pure, moderately active preparation.

Method VIII was designed, with consideration of the results of Method I, to decrease the nitrogen content and to improve the method of evaporation. It was thought possible that, if the group A substance was thermolabile, as considered by many authorities (58, 62), denaturation might have occurred, in Method I, during evaporation to dryness at 37°C. To circumvent this, the material used in Method VIII was evaporated under reduced pressure at a mean temperature of 10 - 13°C. As a result, there was none of the brown discoloration which was observed in the evaporation residue of Method I. The evacuation method of removing the water, chloroform and amyl alcohol was distinctly superior to the incubation procedure, being more rapid and much less destructive to the extracted material. It was found to be unnecessary to use high-vacuum pumps to produce the vacuum required for evaporation; a battery of three water aspirators was found to be entirely adequate and possessed the advantage of being unaffected by the volatile liquids.

The method of Meyer and Palmer (56), used as Method II in this research programme, was also totally unsuccessful in extracting active

material from umbilical cords. The preparation was incompletely dried, nearly insoluble in water and showed brown discoloration similar to that observed in preparation 3. It has not been established why this method failed, but the results of other methods indicated that water alone, especially under refrigeration, was incapable of penetrating the cellular material sufficiently. The Molisch test, which is not specific for carbohydrates inasmuch as glycoproteins give a positive reaction, thus could have indicated the solution of cellular protein material rather than the intra-cellular agglutinogen. It was unlikely that the acetic acid, used to adjust the pH before ethanol precipitation, affected the extract since, in Method III variation (e), the extracted material was treated with a much stronger acid without detriment.

The several methods given the code number 5 were so grouped because, in each case, one or more enzymes were added to the extraction mixture as an aid to cellular penetration.

In the aforementioned paper of Baer et al. (7), pepsin was used which contained, or was mixed with (cf. 7), an equal weight of magnesium sulfate. This type of pepsin was unavailable, so, to approximate the conditions described by the authors (7), nearly equal weights of pepsin and of magnesium sulfate were added to the subdivided cord material, in variations (a) and (b) of Method III. No reason was given for the presence or addition of magnesium sulfate. Pepsin does not require the presence of magnesium as an activator and since it appeared that no specific purpose was served by its addition, later variations omitted the salt.

Because the end point of enzymatic action was judged by the failure of the pH to show a significant rise during any twenty-four hour period, the "Accutint" pH Test Papers were tested with a Beckman pH meter for the

range pH 1.0 - 6.0. It was found that the pH Test Papers were accurate to within 0.3 unit of pH, and that definite color changes were evident with these papers for variations of 0.1 pH. As a result the enzymatic action was considered to have ceased when the pH rose less than 0.1 between two consecutive tests.

Variations (a) and (b) of Method III illustrated the effect of dialysis on the extracted material. The results of variation (a) were in accord with those of Baer et al. (7), who also noted that only a trace of phenol-insoluble material was obtained. The ability of this water-soluble, phenol-insoluble material to dialyse suggested that it had a relatively low molecular weight. The optical activity of the phenol-insoluble moiety 5-B P.I. illustrated its innate difference from preparations 5-A and 5-B. Preparation 5-B P.I., which presumably would have been removed by dialysis, showed negligible ability to inhibit the isoagglutination of A_1 cells, but exerted a powerful inhibitory influence on pseudo-A_2 (or $A_2 > A_1$) cell agglutination.

Only the most readily water-soluble fraction of the initial ethanol precipitate was deproteinized. The group specific material 5-A, obtained after dialysis of the cord extract, was much less soluble in ethanolic solution than 5-B, which did not precipitate from 90 (w/v) % phenol until the ethanol concentration had been increased to 15 (v/v) %. Preparation 5-C showed a similar solubility in phenol-ethanol mixtures. Preparation 5-B contained much less nitrogen than did preparation 5-A, and was much more active than the latter. Material 5-C contained less nitrogen than either 5-A or 5-B, a property reflected in its activity. The preparation was almost 80 times more active than preparation 5-A. In serum dilutions of 1:4 and 1:16 materials 5-B and 5-C failed to inhibit complete-

ly isoagglutination, although the former exhibited a definite tendency to inhibit cellular agglutination. However, in serum diluted 1:64, the activity of preparation 5-C was 10 times greater than that of 5-B. Both preparations were able to cause complete inhibition of isoagglutination in that serum dilution.

Variation (d) of Method III proved that the addition of magnesium sulfate to the enzyme substrate was unnecessary. In addition, a valuable comparison between the heretofore untried deproteinization method of Gerheim et al. (23) and the method of Morgan and King (62), which was used in the preceding variations of Method III, was obtained. The Morgan and King method required five or six days to complete, and the yields obtained were low; it was hoped to find a less time-consuming method that would give comparable results. The method of Gerheim et al. was extremely simple and could be completed in about two days. The yields obtained with this method were greater than with the Morgan and King procedure, and the nitrogen content of the final product was lower. However, in spite of these advantages, the Gerheim et al. method was inferior to that of Morgan and King inasmuch as many of the products showed a marked decrease in activity on purification.

Preparation 5-E was atypical since purification, both by the Gerheim et al. (23) and by the Morgan and King (62) methods, leads to a decrease in activity in some instances. The optical activities of 5-E G and 5-E M-K suggested that these had a similar molecular structure, although it was evident that the Morgan and King method caused less basic changes. The 5-E and 5-E G exhibited identical activities up to a serum dilution of 1:64 when tested with A_1 cells; at that dilution 5-E G caused complete inhibition of isoagglutination but was only one tenth as active

as 5-E in causing partial inhibition. When tested with pseudo-A_2 cells, the detrimental effects of purification became more obvious. The purified material 5-E G, at best, showed an activity equal to that of 5-E, and elsewhere 5-E G was only one tenth as active. Preparation 5-E M-K was tested with A_1 cells only, and was the least active in the serum dilution of 1:4. However, at a serum dilution of 1:16, preparation 5-E M-K was 100 times more active than either 5-E or 5-E G, and at a serum dilution of 1:64, was 10,000 times more active than 5-E G, in completely inhibiting agglutination. Strangely, 5-E M-K was no more active in producing partial inhibition of agglutination in the same serum dilution, than was preparation 5-E. The results indicated that the Morgan and King deproteinization method was superior to that of Gerheim et al. although both methods were proven to be capable of increasing the activity of crude extracts.

It is doubtful if the significant decrease in the nitrogen content (and, accordingly, the significant increase in the purity) of the preparations of Method III, compared to those of Methods I and II, were due to the method of purification entirely. The decrease was more probably the result of the combination of peptic and acid hydrolysis with the selective precipitation from 90% phenol. Such a co-operative scheme of proteolysis and hydrolysis in purification has been suggested by Meyer et al. (57).

The results of Method III, variation (e), illustrated that the blood contained in the cords was responsible for a significant portion of the active material extracted. They proved also that the cord material itself was a source of the agglutinogen, and that blood group substances could be obtained from erythrocytes by peptic digestion. The blood provided the most active material obtained by this method.

Unlike the material obtained in variation (g) of this method,

the final wash waters, containing, presumably, the least soluble blood extracts, were the source of the highly active material 5-F B. The first wash waters, all of which were strongly colored with blood and were much more viscous than the second group, yielded material 100 times less active than preparation 5-F B. Once again some deactivation was observed after purification by the method of Gerheim et al. (23). This was particularly noticeable at low serum dilutions.

After highly active group specific material had been obtained from hog stomach linings by autolysis, the method was repeated as Method VI, variation (a). This method produced material of moderate activity in a moderate yield. However, little degradation of the cord material was observed; no putrefaction occurred as had with the hog stomachs. The nitrogen content of the crude material was abnormally high. It was felt that the conditions present during the autolysis of the stomach linings were not approximated in the preparation of material 8-A, inasmuch as there was an absence of enzymes in the latter procedure while the former almost certainly contained pepsin from the glandular portion of the stomach linings.

As a result of these conclusions, Method III variation (f) was devised to study the combined effects of peptic digestion and autolysis. It was hoped that, over the ten day incubation period, the enzymatic activity would be similar to that in variation (e) of Method VI. The effect of the added enzyme was obvious. The cord material was almost completely digested, and, during autolysis, a persistent rise in pH was observed. This property was insignificant in the preparation of material 8-A.

A clue to the similarity of the final purified preparations was found in the optical activities of 5-8 and 8-A which, although the nitrogen

contents of the preparations were different, were identical within experimental error. The nitrogen content of the crude 5-8 material was nearly 1% less than that of the crude 8-A preparation. However, the Sevag (71) method yielded a purified material with 0.2% less nitrogen than preparation 5-8. Nevertheless, although, as with the Gerheim et al. (23) method, more of the nitrogenous moiety was removed by Sevag's procedure, the final product was less active than that obtained by purification by the Morgan and King (62) procedure.

Bendich et al. (10) had reported that both tryptic and peptic digestions of hog stomach linings were successful in assisting the extraction of active material, but a combination of peptic and tryptic digestion, such as was carried out in Method III, variation (g), had not then been reported. This procedure resulted in the largest yield of crude extract obtained in this research project.

Most interesting results were obtained by fractionation of the water extracts, each of which was treated in an identical manner. The crude material was finely comminuted previous to extraction so that all of the group specific substance would be exposed to the solvent. In this way it was hoped to differentiate the more soluble material from the less soluble. The results showed that the agglutinogen was dissolved in water more readily than other less active material. A continuous, step-wise decrease in activity was observed in going from preparation 5-PT-A to preparation 5-PT-C.

For a given fraction, the nitrogen content decreased with increasing activity, although no inferences could be drawn from this observation. The effect of nitrogen content on activity, if any, was most irregular. For example, with preparation 5-PT-A (cf. Table III) a decrease of 0.1% nitrogen resulted in a tenfold increase in activity, while a further

decrease of 0.5% in nitrogen content resulted once more in increasing the activity 10 times.

Variation (h) of Method III was carried out before any of the other variations in order to test the method on a known source of blood group A substance. The success with which the material was thus extracted proved the procedure to be feasible. Thus, if this same method had failed to extract active material from umbilical cords, it would have indicated that the source, and not the technique, was at fault. The results of this variation were in agreement with those of Kazal et al. (44) who found the glandular lining of a hog stomach to be a better source of the agglutinogen than the non-glandular lining. The glandular linings were found to be superior both in yield and activity of extracted material.

The method of Meyer et al. (57), listed as Method IV, was the most rapid method attempted, because extraction was carried out at a relatively high temperature but for a very short time. No degradation was evident as a result of warming to 70°C. The nitrogen content was not unusually high, emphasizing the importance of hydrolysis in the removal of the nitrogenous moiety, as suggested by other investigators (57).

Purification by adsorption with kaolin from acid solution was attempted for the first time, and was only moderately successful. The purity of the final product was increased by collecting the material which was least soluble in ethanol solutions. Further hydrolysis was effected by precipitation, in the cold, with dilute acetic acid. The protein moiety was reduced more by precipitation with a heavy metal, in this case lead. Notwithstanding, this protracted series of deproteinizations removed only 0.3% of the nitrogen. The yield of the final product, preparation 6, was excellent, but the activity was low, and was not increased by

purification.

The variations of Method V differed from those of Method III by controlling the pH through the use of buffer solutions, rather than by daily adjustment. The results indicated that the continuous rise of the pH of the buffer solutions, during the five day incubation periods, hampered the enzymatic activity and resulted in a serologically inferior product.

Regardless of the nitrogen content of preparation 7-A, the amount of inert material in the crude extract was very large, resulting in an apparently excellent yield; however, the material extracted showed almost no blood group activity. Most of the inactive impurities were removed by the Morgan and King (62) deproteinization method, resulting in the greatest decrease in yield observed in the research programme. As there was a decrease of only 0.4% in the nitrogen content, much of the contaminating material removed must have been non-nitrogenous in nature.

Preparation 7-B was extracted from undried umbilical cords, and during purification was fractionated into two portions, one soluble in aqueous ethanol-acetone solutions but insoluble in ether solutions; the other, insoluble in ethanol solutions. The former material was never obtained from dried umbilical cords. This preparation, 7-B-2, was found to be insoluble in 95% ethanol and in pure acetone, but very soluble in water. Preparation 7-B-2 exhibited strong activity, being more active at low serum dilutions than the more typical preparation, 7-B-1. Again, purification by the method of Morgan and King (62) was shown to yield material of increased activity. There was no indication of degradation of the purified material.

Those preparations given the code number 8 were so grouped because each was obtained through autolysis of the source material.

It has been stated (cf. ante) that prolonged incubation of the source material without frequent adjustment of the pH resulted in inferior products. Variations (b) and (d) of Method VI were carried out specifically to study this observation and indicated that it was true.

Material 8-B was obtained after ten days of uninterrupted autolysis, during which time no control was exerted on the pH, while preparation 8-D was extracted from umbilical cords which were readjusted to pH 2.3 halfway through the incubation period. The results indicated that the crude 8-B material, while in far greater yield than preparation 8-D, was much less nearly pure. On purification, an extremely large decrease in the yield of 8-B was observed. Judged by their abilities to completely inhibit isoagglutination, preparation 8-B was consistently more active than 8-D, which, obtained in approximately twice the yield of 8-B, was only one half as active in this respect. However, considering the respective abilities of preparations 8-B and 8-D to cause partial inhibition of isoagglutination, the activity of the latter was found to be at least 5 times greater than that of 8-B.

Variation (c) of Method VI was devised to test the completeness of the extraction of the agglutinogen by the usual methods. The results showed that the initial autolysis solution contained about 80% of the extracted material. Each of the ensuing autolytic extractions removed increasingly smaller amounts of the material.

The first and third extractions yielded preparations of extreme activities, while the second extraction produced only moderately active material. In all cases, the activity paralleled the nitrogen concentration. Preparation 8-C-3 was the most active, and had the lowest nitrogen content, while the pure preparation 8-C-2 was the least active and had a

nitrogen content approaching that of the crude 8-C-1 preparation.

Material 8-C-3 was obtained after twenty days of incubation. The first ten days of incubation were not interrupted for pH control. Afterwards, the old buffer was filtered off and a fresh solution of identical pH was added after each five days of incubation. As a result, the combined effects of variations (b) and (d) were exerted upon preparation 8-C-3, which was found to be the most active material prepared in this research project, and which was, with respect to activity, equal or superior to all but three preparations reported in the literature. The preparation was obtained in a yield of 0.9% by weight, and completely inhibited agglutination of group A_1 erythrocytes, that is, it completely neutralized all of the anti-A in the B serum, in dilutions of at least 1 part in 10 million. These results were obtained with serum diluted 1:16 and 1:64.

Preparation 8-C-1B, when pure, was less active than 8-C-3 only in the lower serum dilutions, but equalled the activity of preparation 8-C-3 in the higher serum dilutions. The optical activities of these two preparations were equal, within experimental error.

Method VI, variation (e), was carried out as a control, to test the practicability of the method on a known source of active material. The extract, preparation 8-Hg, was obtained in good yield, and showed moderate group A activity. Its properties concurred with those of other such materials reported in the literature (14, 15, 31, 57, 62), being laevorotatory, and having, in the purified state, a nitrogen content in the region of 6%.

Material 8-Hg-B, prepared in the same way as preparation 8-Hg except that the hog stomach was undried, was much more active than the latter. It contained 0.1% more nitrogen than 8-Hg, but had a significantly smaller optical rotation.

Variation (f) of Method VI confirmed the superiority of the undried umbilical cords over the dried cords. Using a phosphate-citric acid buffer instead of the usual citrate-HCl buffer, a highly active preparation was obtained. Material 8-Hg-A, while much less active than preparation 8-Hg-B, still was 10 times more active than preparation 8-Hg. The optical activities of 8-Hg-A and 8-Hg-B were equal within experimental error, although the latter contained much less nitrogen. The results once again suggested that some nitrogen was essential for optimal activity.

The method of Bray, Henry and Stacey (14), used as Method VII, failed to extract active material from umbilical cords. Only a trace of activity was observed in preparation 9-b, although its activity was 10 times greater than that of preparation 8-b. The former preparation was in accord with the findings of other investigators (15, 44, 79, 80) with respect to optical activity and nitrogen content.

In the above methods of extraction, the preparations were usually subjected to dessication either by storage in vacuo over P_2O_5, or by solvent exchange; the latter was found to be distinctly superior in every way. Drying with P_2O_5 was a slow, inefficient method of drying the preparations and often resulted in their discoloration. The drying required not less than 18 hours and the final product was often contaminated with traces of P_2O_5 or droplets of phosphoric acid.

Drying by solvent exchange was found to be a very rapid method which yielded a very dry, uniform, uncontaminated product. Only small amounts of the solvents were required, 75 ml. of 95% ethanol, 50 ml. of acetone and 20 ml. of ethyl ether being sufficient to dry 2 gm. of preparation. Only a few hours were required to complete the dessication. The solvents were drawn through the preparation, which was collected in a

Buchner funnel, separated by one or two water aspirators and collected in a 1 litre filter flask. The last traces of the solvents were removed by drawing air through the preparation for ten to fifteen minutes. In the initial stages of drying, it was found advantageous to discontinue pumping for a few minutes whenever frost formed in the filter flask.

SUMMARY

Methods for the isolation and purification of the human blood group A, B and O substances from umbilical cords and of the hog blood group A substance from hog stomach linings have been described.

Umbilical cords have been proven to be a source of a blood group A substance of very high activity, as measured by isoagglutination inhibition. No active material was extracted from group B or group O umbilical cords.

The A substances from umbilical cords were found to be dextrorotatory polysaccharide-amino-acid complexes. The most active blood group A substance, preparation 8-C-3, was obtained from variation (c) of Method VI, which was based upon the autolysis procedure of Bendich, Kabat and Bezer (10).

Umbilical cords kept in an undried frozen condition were proven to be better sources of group specific materials than those stored and dried under acetone or ethanol.

The deproteinization method of Morgan and King (62) was found to yield consistently superior results to that of either Sevag (71) or Gerheim, Berkut and Gerheim (23).

The solvent exchange method of drying the purified preparation was shown to be faster and more efficient than drying over P_2O_5 *in vacuo*.

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