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A STUDY OF MECONIUM

AND

BLOOD-GROUP-SPECIFIC SUBSTANCES

A dissertation submitted to the

Graduate School of Arts and Sciences
of the University of Cincinnati

in partial fulfillment of the
requirements for the Degree of

DOCTOR OF PHILOSOPHY

1951

by

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A STUDY OF MECONIUM
AND
BLOOD-GROUP-SPECIFIC SUBSTANCES

I. Introduction

Meconium (1,2) is the very characteristic first stool of a newborn infant. It is a viscid, sticky, odorless, and greenish-brown to black material. A certain amount of liquid is absorbed from the meconium during development, causing the meconium to become increasingly firm, solid, and dark, as gestation proceeds.

Meconium is regularly seen during the fifth month of fetal development, but has been observed as early as the fourth month. At this time it is lighter in color because biliverdin is not formed in significant amounts before the sixth month.

At birth the amount of meconium varies from sixty to as much as two hundred grams, and normally some is passed within four or five hours after birth. After several meconium stools, the fecal material becomes transitional--nonhomogeneous, thin, sour, slimy, and brown to green in color. This consists of remnants of material from the meconium stool and incompletely digested elements from the milk feedings. The transitional stool gradually acquires the golden yellow, pasty, homogeneous, acrid smelling characteristics of the milk stool.

An early theory of meconium formation was that of Tiedemann and Gmelin (3) who believed that the bile and not amniotic fluid or vernix caseosa were essential for the formation of meconium because it was "found in monsters without head or nose."

The later theories of meconium formation have stated that it results from a swallowing of the fetal environmental material (amniotic fluid), collection of cells from the upper alimentary tract, mouth, and skin, fatty material from the vernix caseosa, hairs from the lanugo, enzymes, and traces of blood from unrecognized sources.

Although many people have investigated the composition of meconium, the information available is conflicting and incomplete.

Sheldon and Ramage (4) did a spectrographic analysis of the metallic content of meconium which demonstrated the presence of calcium, sodium, potassium, copper, magnesium, manganese, and iron. The lines for manganese and copper were strong while the iron line was relatively weak.

The phosphorus content of the meconium from different parts of the alimentary tract of fetuses at different ages was measured by Parat and Delaville (5). They concluded that in the small intestine the meconium is a source of nourishment from the third to the eighth month, but that in the large intestine it is merely an unusable excretion.

Meconium has a slightly acid reaction (6), the average being pH 6.1.

Sobotka (7) stated that meconium contains in addition to ash, lipids, cholesterol, cholestanol, and purines, up to 1% bilirubin and biliverdin, which cause the greenish-black color, and taurocholic acid, possibly accompanied by free taurine. Between 60-150 mg. % bile acids were present in meconium.

Bile secretion (8,9) begins in about the sixth fetal month. Most of the bile pigments in meconium are biliverdin and only occasionally bilirubin. Stercobilin never occurs and only appears in feces several days after birth.

A specific color reaction for cholesterol in an ether extract of meconium was obtained by Haussler and Brauchli (10). Schönheimer (11), et al., found that 7% of the total cholesterol in sterile meconium in human embryos was dehydrocholesterol.

An ether extract of meconium also showed a high oestrus titer (12).

The various hormones which are secreted in late fetal life are present in meconium. Cathepsins (13) have been demonstrated in the meconium of immature infants. The intestinal mucosa is very highly developed, and there is evidence that the infant has glands of the Lieberkuhn type scattered among the ordinary mucus glands of the colon, although no

such secretory structures are present in later life. There is no evidence to indicate the lack of protein, sugar, and fat splitting enzymes in the intestinal juices at birth, even when the infant is born a month or more prematurely. Pancreatic amylase and perhaps lipase are reported not to be very active the first few weeks after birth.

Bacteria are absent from the alimentary tract of the fetus and of the infant at birth. However, they appear in the alimentary tract within a few hours after birth. Hall and O'Toole (14) found that the first meconium stools which were passed within ten hours after birth contained culturable organisms in about 40% of the series tested. When the specimens were passed within five hours after birth, 87% of those investigated were sterile. All infants were found to have bacteria within 24 hours after birth.

Blood group substances, with the same serological activity as the red corpuscles possess, can not usually be demonstrated in feces. However in 1933 Witebsky and Satoh (15) mentioned that a Japanese, Yosida, had found blood group substances in meconium.

Sekimoto (16,17) showed that some meconium contained large amounts of these materials while there was very little in others. He separated the infants into secretors and non-secretors and believed that transition types might also be present.

Another Japanese investigator, Okajima (18), showed that the amount of blood group substance diminished rapidly after the first few days of life.

Ureyama (19) demonstrated the presence of a type T substance in meconium.

A meconium plug (20)--a slightly inspissated round or cylindrical mass which is formed in the rectum during fetal life--has a grayish white to pale yellow color and a putty-like consistency compared to normal meconium. However this plug is generally of no practical importance as the subsequent stools are normal after it is passed.

In rare instances the meconium may be tough and inspissated throughout the entire intestine. It is so sticky and mucilaginous that it cannot be propelled by normal peristalsis, and obstruction occurs. In some cases the inspissated meconium occurs as a result of stenosis of the ileocecal valve or volvulus. If the obstruction of the bowel can be relieved, no further difficulty is usually encountered.

Even more viscid and mucilaginous is the meconium found in cases of meconium ileus. Here a mass of meconium fills the terminal 10-20 cm. of the ileum and is apparently unable to pass the ileocecal valve. The ileum proximal to this becomes distended, and oftentimes this obstruction causes intestinal perforation, and meconium peritonitis results.

As early as 1905 Landsteiner (21) thought that the cause of this condition was due to an error in metabolism due to the absence or too late secretion of pancreatic juice or bile which caused a failure of fat and protein digestion of the meconium and caused the contents to thicken. Perhaps the glands of the gastro-intestinal tract also failed to secrete.

The meconium plug does not contain bile pigment or cholesterol, and some people have assumed that if this condition were prolonged, the meconium would become thick, greasy, putty-like, and would lead to intestinal obstruction.

Dodd (22) stated that in all cases in which complete autopsy with histologic studies had been done on patients with meconium ileus, either the liver or bile ducts, or the pancreas was found to be pathologic.

Lack of biliary secretion is not a rare anomaly, but does not always result in meconium ileus. Several cases of patients with normal pancreas but with cystic livers have been reported in which the meconium was said to have had a putty-like consistency. However Farber believes that microscopic lesions and obstructions of the acini of the pancreas were not recognized in these cases. Probably the failure of pancreatic secretion or bile to reach the gastro-intestinal tract during fetal or neonatal life is at least part of the cause of meconium ileus.

Farber reported finding that meconium which was so sticky and mucilaginous that it caused intestinal obstruction was accompanied in every case by an obstructive lesion in the pancreas characterized by inspissation of the secretions, dilatation of ducts, atrophy of acini, and fibrosis of the pancreas. He found that when this type of abnormal meconium was placed in a test tube with a saline solution of pancreatin, which varied from 1% to 10% in strength at a temperature of 37.5° C., it was reduced within a short time to a semifluid or liquid state. A 1% solution of pancreatin brought the meconium ileus to a semifluid state in two to four hours, while a 5 to 10% solution only required one to two hours. He therefore suggested that meconium ileus might be washed from the bowel by means of an enterostomy with pancreatic extracts used as a solvent.

In several cases no evidence for tryptic activity could be found in the duodenal drainage from patients with meconium ileus.

Those patients with meconium ileus who do survive usually die of suppurative bronchitis, thus indicating that meconium ileus is the first and most severe form of cystic fibrosis.

Not only are obstructive lesions of the pancreas found in newborn infants with meconium ileus, but also in infants

under a year of age who have chronic pneumonia and severe nutritional failure and in older children who have a type of nutritional failure resembling idiopathic celiac disease. In all these cases pancreatic achylia appears to be a primary cause of the symptoms in the intestinal tract.

In the majority of the patients, where congenital malformation of the pancreatic duct is not the cause of the pancreatic achylia, this appears to be a systemic disease involving secretion-producing structures in many parts of the body. Hypoplasia of the gall bladder with atresia of the cystic duct or occlusion of a patent duct by inspissated secretion and also occlusion of the hepatic ducts with similar material have been encountered. Eosinophilic concretions have been found in glands of the intestine, bronchi, and salivary glands. This disease, which is congenital and familial, is called cystic fibrosis of the pancreas (23).

The lungs often show chronic bronchitis which is superimposed by an acute suppurative process, and the bronchial tree often becomes filled with a thick, viscous mucopurulent material. The lungs may show obstructive emphysema, and sometimes atelectasis. Frequently there are signs of vitamin A deficiency.

There is inadequate protein, fat, starch, mineral, and vitamin utilization due to pancreatic insufficiency (24). Normal patients have a duodenal drainage that is clear, non-viscous, and larger in amount that those of patients with cystic fibrosis, in which it is thick and sticky. Normal tryptic activity is above 200 units per cc. of fluid while in pancreatic fibrosis the activity is close to 0. Amylase and lipase activities are also less, but gastric secretions are usually normal in quantity, acid content, and pepsin.

The fact that no two animals or plants are exactly alike has been recognized for many years, mainly because of morphological differences. Toward the end of the nineteenth century, biochemical differences also became evident through the occurrence of violent and often fatal reactions to the transfusion of animal blood from one species to another and even between individuals of the same species.

In 1900 Landsteiner (25,26) made the first observations on the differences between the bloods of normal individuals belonging to the same species. By use of the isoagglutination reaction, he divided humans into three distinct groups. A fourth group was discovered two years later.

Von Dungern and Hirszfeld discovered subgroups in two of the four human blood groups in 1911, and Landsteiner and Levine (27) discovered three more additional properties,

designated as M, N, and P in 1927. Other serological differences have been demonstrated more recently, the most important of which is the Rh factor which was described by Landsteiner and Weiner in 1940.

The presence of these factors in animals is determined by heredity and is not influenced by environment.

The basis for the division of human blood into four main divisions is the fact that human blood contains antigens, agglutinogens, associated with the erythrocytes and antibodies, agglutinins, in the plasma. Both the agglutinogens and the agglutinins can react with specific agglutinins and agglutinogens in the blood of individuals of other blood groups.

According to Landsteiner, the blood group-specific substances A and B, the agglutinogens, may be present in the erythrocytes either singly or together, or both may be absent. Normal human serum contains two different isoantibodies or agglutinins, α and β, and their presence depends upon the agglutinogen in the cells. These agglutinins have the power to combine with certain agglutinogens and cause agglutination of the red blood cells.

Agglutinin α (anti-A) can combine with blood group substance A and agglutinin β (anti-B) with blood group substance B. The blood group substance and the agglutinins found in an individual blood are always mutually compatible.

The combinations that exist naturally are shown in Table I.

Table I

Blood Type	Agglutinogen in Red Cells	Agglutinin in Serum	Cells Agglutinated by
O	---	$\alpha + \beta$	---
A	A	β	α
B	B	α	β
AB	A + B	---	$\alpha + \beta$

There is also an agglutinogen O which reacts with neither α nor β and has no agglutinins occurring naturally in serum. Very rarely a serum is found which does contain agglutinins which are capable of causing specific agglutination of type O cells. More frequently when it is desired to cause agglutination of type O cells, sera prepared from cattle or other animals, which possess that property, are used.

The specific agglutinogen-agglutinin reactions are used for blood grouping and in cross-checking bloods to determine if they are compatible for transfusions. There are two main types of incompatibility: 1) the patient's plasma agglutinates or hemolyzes the donor's cells, a reaction which is likely to be fatal to the patient; 2) the donor's plasma agglutinates the patient's cells, but the patient's plasma

does not agglutinate the donor's cells. This second type of transfusion has generally been found safe because the donor's plasma is so diluted that it cannot cause agglutination. People belonging to blood group O have been called 'universal donors' because their blood cells cannot be agglutinated by other plasma.

In Britain and America about 45% of the population belongs to blood group O, 40% to A, 10% to B, and 5% to AB. However the percentages vary slightly according to the race.

The occurrence of substances possessing blood-group-serological activity in cells other than erythrocytes was discovered by Landsteiner and Levine in 1926. Later supporting evidence of their presence in numerous tissues and secretions was obtained. In 1931, Schiff reported that blood group specific substances could be extracted from tissues in a water-soluble form and a form soluble in alcohol and other organic solvents. Water soluble substances were also found in body fluids, such as saliva, urine, digestive juices, and so on.

Every individual does not secrete these blood group substances in the body fluids, Schiff and Sasaki designated those which do possess the property of secreting blood group substances as secretors and those which do not as non-secretors. The ability to secrete these substances is hereditary, and this ability is transmitted as a simple

Mendelian dominant which is in no way linked to the presence of any of the agglutinogens.

The Rh factor was originally believed mainly restricted to the blood cells, but Witebsky (28) in 1945 demonstrated the presence of Rh factor in amniotic fluid and other workers have found traces in saliva. It is possible that the methods used to demonstrate its existence were not sufficiently sensitive. The M and N factors likewise appear to be mainly restricted to the erythrocytes.

The blood-group-specific substances possess the ability to inhibit isoagglutination, to inhibit the hemolysis of sheep erythrocytes by rabbit sera under certain conditions, and to participate in precipitin reactions with the homologous agglutinins.

At first the investigations with these materials were largely concerned with their serological activity, but recent investigations have been aimed at the determination of their chemical nature.

The erythrocytes were assumed to contain serologically active substances in their stroma, but studies on the red cells have been largely unsuccessful. Several workers showed that a specific substance could be extracted with alcohol from the red cells, and in 1934 Hallauer (29) claimed to have extracted with alcohol a serologically active material from human red cells of blood groups A, B, and O. He

did show that active material contained carbohydrate and had an elementary composition in the order of 6.8-7.0% nitrogen, 43-46% carbon, 7.0-8.5% hydrogen, and 15.2% phosphorus, but could not draw any definite conclusions about its chemical nature.

Recently Kosjakow and Tribulev claimed to have isolated A, B, M, and N factors from red cells by aqueous alcoholic extraction, deproteinization, dialysis, and precipitation of the active material with 96% alcohol. However this work has not been confirmed.

Although the relationship between the blood group substances 'proper' from the erythrocytes and materials with blood group activity found in body fluids is not clear, the latter have provided a more accessible source of material for investigation. The relationship may be no more than a close similarity in chemical structure of some parts of the molecular complex, but the study of one may at least lead to a partial understanding of the other.

These blood group specific substances from human sources, however, are surprisingly hard to obtain in sufficient amounts in a purified form. About 15 mg. of purified substance can be obtained from 500 cc. of saliva, but it is very difficult to obtain this much saliva from one person. Another complicating factor is the presence of enzymes in the saliva which destroy the activity.

Assuming 100% yield, 250 liters of urine would provide only 5-10 mg. of purified substance. The yields from gastric juice are sufficiently high to make the accumulation of preparations in useful amounts possible. Approximately 20-125 mg. of purified substance can be obtained from one liter of amniotic fluid.

Morgan and co-workers (30,31) obtained a very active blood group substance from cystic fluids.

Although blood group activity has been found in these and other body fluids, they have not been convenient sources of material in large enough amounts for adequate research. Therefore other materials which exhibited blood group activity such as pepsin, hog stomach, hog mucin, abomasus of cattle, horse saliva, and peptone have been of special value.

No complete chemical investigations of the structure of blood group substances have been reported, but many workers have partially investigated these materials. They appear to be predominantly carbohydrate in nature. D-galactose and N-acetyl-D-glucosamine have frequently been found as constituent sugar units. L-fucose has also been found in several cases, and D-mannose in a few. The arrangement of the sugar units within the carbohydrate itself is still uncertain.

Bray, Henry, and Stacey (32) have done considerable work toward the partial elucidation of the arrangement. By methylating the polysaccharide, prepared from commercial pepsin, they found that part of the fucose was undoubtedly present as an end group originally while some of it existed as intermediate links in the molecule. A small proportion of the D-glucosamine units was also linked as terminal groups, while the rest may constitute a type of polyglucosamine 'core'.

In studying the material purified from gastric juice, Bray, et al. (33), found that these substances differed from those obtained from pepsin since the methyl ethers which were obtained had different specific rotations.

Morgan (34) found that when blood group substances were treated with 0.1 N sodium carbonate at 100° C. there was some degradation of the molecule for some glucosamine residues were split off, the serological activity was reduced, and the rotation changed from $[\alpha]_D$ + 10° to - 20°. It would therefore appear that some of the hexosamine units were linked to the remainder of the complex through position 1 and that this link was very unstable to dilute alkali.

In a study of blood group substances from hog stomach linings, Kabat, et al. (35) found the material serologically stable for two days at 37° C. between pH 1.07 - 10.7. Heat

treatment for two hours at 100° C. did not cause any loss of activity if the pH was between 2.97 - 7.58. Inactivation did occur at 100° C. at pH values below 1.03 or above 9.03.

When they (36) exposed blood group substances in dilute HCl to 100° C., A and B activity was destroyed in 1-2 hours. 60-80% of the fucose and some amino acids were made dialyzable.

Freudenberg and Eichel (37) reported that they were able to restore the activity of the substance by reacetylation after it had been inactivated by treatment with alkali.

It is now recognized that amino acids are important constituents of the blood group substances. Landsteiner and Harte (38) demonstrated that most of the non-hexosamine nitrogen was accounted for as amino acid nitrogen. Freudenberg, Walsh, and Molter (39) were able to isolate threonine from blood group substance A.

Morgan (34) reported that at least fifteen amino acids, including threonine and hydroxyproline in high concentrations, were present in blood group substance. Cystine was absent.

Brand and Saidel found the following constituents in blood group substance A from hog stomach (40), and material from horse gastric mucosa with both A and B activity (41):

Constituent	Blood Group Substance From Hog Stomach %	Blood Group Substance From Horse Gastric Mucosa %
Reducing Sugar as Glucose	56.0	37.0
Glucosamine	30.4	14.3
Total Nitrogen	6.1	6.0
Glycine	1.6	0.6
Valine	0.7	0.6
Isoleucine	0.3	0.2
Proline	3.3	0.9
Phenylalanine	0.1	0.3
Tryptophan	0.2	0
Histidine	0.6	0.6
Lysine	1.0	0.7
Aspartic Acid	0.8	1.6
Glutamic Acid	1.3	2.8
Serine	1.9	2.0
Tyrosine	0.3	0.2

In the material from hog stomach, these results accounted for 66.1% of the total nitrogen.

The mixed enzyme preparations from Clostridium welchii (42) inactivated A and O blood group substances isolated from gastric mucin. This was accompanied by an increase in free amino nitrogen, liberation of free α-amino acids, increased reducing power, increased levorotation, and decreased viscosity. Therefore it seemed possible that the protein moiety was concerned either alone or together with the carbohydrate moiety in determining the activity and specificity of substances.

The work that has been done on blood-group-specific substances from human sources is summarized in Table II and from animal sources in Table III.

Table II

BLOOD-GROUP-SPECIFIC SUBSTANCES FROM HUMAN SOURCES

Source	N %	C %	H %	Amino Acid N %	Reducing Sugar %	Hexos-amine %	Acetyl %	Fucose %	$[\alpha]_D$
Erythrocytes (29)	6.8-7.0	43-45	7.0-8.5	---	---	---	---	---	---
Urine				about					
Type A (37,43)	5.0-5.5	43-45.5	5.9-6.5	0.5	---	---	9.1	---	-5° to $+5^\circ$
Type O (44)	3.8	43.4	5.9	---	---	---	7.9	---	-21°
Gastric Juice									
Type B (45,46)	1.5-1.6	---	---	---	75.0	---	---	---	---
Type O	2.8	---	---	---	39.8	---	---	---	---
Type B (non-sec.)	2.73	---	---	---	30.3	---	---	---	---
	0	---	---	---	76.2	---	---	---	---
Type A (33)	2.76	---	---	---	78.0	---	---	---	$+65.5^\circ$
Type B	2.22	---	---	---	82.3	---	---	---	$+81.1^\circ$
Type O	1.75	---	---	---	83.5	---	---	---	$+70.2^\circ$
Saliva									
Type A (47)	5.65	---	---	2.48	45.5	23.3	---	---	---
Type B	5.33	---	---	2.35	48.5	21.7	---	---	---
Type O	5.74	---	---	2.91	46.5	21.5	---	---	---
Type A (48,49)	3.6-7.7	---	---	---	47-64	21-34	7.2-10.3	11-16	-2.5° to -21°
Type A_2	5.5-6.1	---	---	---	54-60	26-30	---	16.4	-27.5° to -37°
Type B^2 (50)	3.3-5.3	---	---	---	45-68	19-30	7.7-13	11.6-13.0	Av.-20°
Cyst Fluids									
Type A (30,51)	5.4-6.0	44-45	6.7	2.5	50-58	27-34	10.0	18.0	$+11.0^{\circ *}$
Type O (52)	5.9	44.5	6.6	2.5	48	38	---	---	$-23.0^{\circ *}$
Amniotic Fluid									
Type A (49)	4.2-5.5	---	---	---	48-50	22-25	---	9.6-13.3	-20.0°
Stomachs									
Type A (49)	5.3-6.8	---	---	---	45-51	25-28	---	9.0-16.0	-2.5° to -5°

* 5461 A°

Table III

BLOOD-GROUP-SPECIFIC SUBSTANCES FROM ANIMAL SOURCES

Source	N %	C %	H %	Amino Acid N %	Reducing Sugar %	Hexosamine %	Acetyl %	Fucose %	$[\alpha]_D$
Peptone (53)									
Difco	5.85	46.7	6.53	---	73	---	9.56	---	+11.5
Pfanstiehl	5.48	44.9	6.23	---	62	---	8.9	---	+ 9.6
Hog Pepsin (54)	6.2	46.9	6.6	---	70.7	27	10.0	---	+16.0
Hog Gastric Mucin (55)	5.7-6.4	44.8-45.9	6.4-6.6	2.2	43-50	24-27.3	9.2-11.5	---	+8° to +14°*
Hog Mucin (56)	3.3-6.2	---	---	---	---	30.8-38.6	9.2-16.8	---	-8° to +8.7°
Hog Stomach (38, 36,57)	5.5-6.3	46-47	6.6-6.9	---	50-60	29-33	10.5-12.0	---	+19.8° to + 21.9°
	5.6-6.8	---	---	---	46-59	25-32	---	6.6-9.9	---
	5.7-6.6	---	---	---	55-61	30-34	9.1-11.3	---	---
Horse Saliva (58)	7.1	44.6	6.9	---	56-58	---	9.4	---	+10.0°
Horse Stomachs									
Types A and B (41)	6.0	---	---	---	37.0	14.3	---	---	---
Type A (50)	4.0-7.4	---	---	---	42-54	8-27	---	1.8-4.9	---
Type B	6.1-7.1	---	---	---	46-53	15-29	9.1-9.4	4.7-9.7	---
Minimal A or B Activity	7.0-7.1	---	---	---	42-52	19-27	---	3.2-4.3	---
No Activity	5.5-8.0	---	---	---	40-52	11-27	8.4	1.7-4.7	---
Cow Stomach (59) (Abomasus)	5.0-7.2	---	---	---	51-60	23-34	---	1.5-5.2	---

* 5461 A°

There is considerable controversy between the various molecular weight determinations. From solubility and diffusion rates Westphal, Marriott, Groenewoud, and Molter (60) concluded that it was a neutral polysaccharide with a molecular weight of about 1000. However later Freudenberg, Molter, and Walsh (61) found the molecular weight of A material from sperm was about 10,000 in osmotic pressure experiments.

Meyer (62), in 1945, stated that the estimated molecular weight of blood group substances by sedimentation rate in an ultracentrifuge was at the minimum, 40,000, and by osmotic pressure experiments between 70,000 and 200,000.

Blood group A and B substances are antigenic in man (63), the A substances causing the formation of α-iso-agglutinin when injected in persons of blood groups O and B, and the B substances causing the formation of β-iso-agglutinins when injected in persons of blood groups O and A.

The purified blood group substances have acquired considerable commercial importance. In order to avoid transfusion reactions due to the presence of high isoagglutinin titres in some type O bloods, blood group substances can be added to neutralize the isoagglutinins (64). Usually blood group substances from animal sources are used, However, it is very difficult to free these substances from traces of protein which may cause anaphylactic reactions upon injec-

tion. Therefore there is a definite need for an adequate source of blood group materials from human sources, since these substances would be less likely to cause anaphylactic shock upon injection.

Blood group substances are also used to remove the agglutinins α and β from sera which are to be used for the determination of other blood factors found in humans such as Rh, M, and N.

Although blood group substances are found in meconium, they are soon destroyed in feces by a blood group enzyme which attacks the water-soluble blood group specific material. Witebsky and his co-workers (15,65) found blood group specific substances in the jejunum and upper ileum. A marked decrease in amount occurs in the lower ileum and caecum while the colon and rectum of adults are free of substance. The blood group destroying power first appears in the lower ileum or caecum and its effectiveness increases in the lower parts of the large intestine. It has not been determined whether this enzyme is formed by the body or the bacteria growing in the intestinal tract.

II. Methods

This research project required the use of a large number of chemical and serological methods, which are discussed below with the modifications necessary for their application to the materials analyzed.

A. Chemical Determinations.

1. Reducing Sugar Determination. The photometric adaptation of the Somogyi reducing sugar method which was devised by Nelson (66) was used for this determination. The sample to be analyzed is hydrolyzed with a mineral acid, and then carefully neutralized to the phenolphthalein endpoint with sodium hydroxide, and the solution made just acid with 0.1 N hydrochloric acid. Proteins can be precipitated with the Somogyi reagents, zinc sulfate and barium hydroxide, if they are present and might cause interference with the reducing powers of the solution. The test depends upon the reduction of copper sulfate by reducing sugars in an alkaline medium; the reduced copper in turn will reduce a solution of arseno-molybdate to form a blue color-complex.

Reagents: a. 5% $ZnSO_4 \cdot 7H_2O$.

b. Approximately 0.3 N $Ba(OH)_2$ was prepared by dissolving 48.8 g. of $Ba(OH)_2$ in a liter. This was stored in a

bottle protected with a soda lime tube. The barium and zinc solutions were adjusted so that 5 cc. of zinc required 4.7-4.8 cc. of barium to produce a definite pink to phenolphthalein with the zinc diluted to 25 cc. with water.

c. Copper Reagent A. 25 g. anhydrous sodium carbonate, 25 g. Rochelle salts, 20 g. sodium bicarbonate, and 200 g. sodium sulfate, anhydrous, were dissolved in water and diluted to one liter. The solution was filtered if necessary. It was stored at room temperature.

d. Copper Reagent B. 15% $CuSO_4 \cdot 5H_2O$ containing 1-2 drops of concentrated sulfuric acid per 100 cc.

e. Arseno-molybdate reagent was prepared by dissolving 25 g. of ammonium molybdate in 450 cc. of distilled water and adding 21 cc. of concentrated sulfuric acid. This was mixed well, and 3 grams of $Na_2HAsO_4 \cdot H_2O$ in 25 cc. of water were added. This was then incubated 48 hours at 37° C. or heated slowly to 55° C. for 25 minutes, stirring constantly. It was stored in the dark.

Procedure:

In order to determine the amount of free reducing sugar, 5 cc. of the sample to be tested were treated with 1 cc. of $ZnSO_4$ and 1 cc. of $Ba(OH)_2$. These were carefully mixed and centrifuged. Appropriate samples of the supernatant were analyzed for reducing sugars directly.

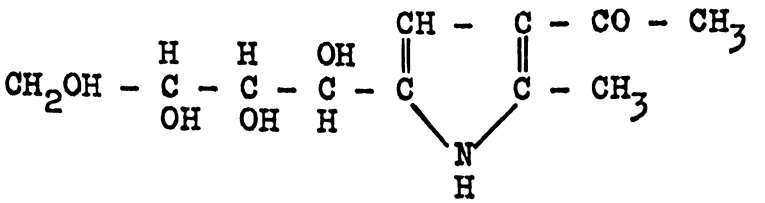
To determine the total amount of reducing sugar, the sample to be tested was hydrolyzed in one normal mineral acid on a boiling water both for two to four hours, using an air reflux condenser to prevent evaporation. After cooling. the mixture was carefully neutralized to pH 7 with sodium hydroxide, using phenolphthalein as an indicator, and just removing the pink color with 0.1 N hydrochloric acid. One cc. $ZnSO_4$ and one cc. $Ba(OH)_2$ were added, and the mixture diluted to a definite volume and centrifuged. Appropriate samples of the supernatant were analyzed for reducing sugars. Later it was found that treatment with the deproteinizing agents was unnecessary with these materials.

For the determination of the amount of reducing sugar, 1 cc. of the sample to be tested or a smaller sample diluted to 1 cc. was treated with 1 cc. of the copper solution (prepared from the stock solutions by mixing 1 cc. of Copper Reagent B with 25 cc. of Copper Reagent A) in constricted sugar tubes. A stopper or glass cap was put on the tube to reduce the contact with air and help prevent oxidation. The mixture was then heated on an actively boiling water bath for twenty minutes, and then cooled in a cold water bath for three minutes. One cc. of the arsenomolybdate solution was added, and the mixture thoroughly shaken so that all of the carbon dioxide evolution was complete before diluting to

25 cc. with water. The sample was thoroughly mixed and read in a Coleman Spectrophotometer at 520 mμ. After dilution the color was stable for at least twenty-four hours.

A blank determination on a cc. of distilled water and an appropriate series of standards, usually glucose, were run with each set of determinations. The most accurate range was with 50-150 μg. of reducing sugar.

2. Amino Sugar Determination. The modification of the Elson and Morgan (67) method for the determination of amino sugars as described by Kabat and Mayer (68) gives reliable results even in the presence of protein. The amino sugars are freed by mild hydrolysis with a mineral acid, and the hydrolyzate is carefully neutralized. The amino sugars react on heating in an alkaline solution with acetylacetone to give 3-acetyl-2-methyl-5-(tetrahydroxybutyl)-pyrrole:



The pyrrole condenses with p-dimethylaminobenzaldehyde to give a characteristic color which may be read photometrically.

Reagents: a. The acetylacetone solution was prepared immediately before use by dissolving 0.2 cc. of acetylacetone in 10 cc. of 0.5 N sodium carbonate. The acetylacetone, which should be colorless, was kept cold.

b. Ehrlich's reagent was prepared by dissolving 0.8 g. of p-dimethylaminobenzaldehyde in 30 cc. of aldehyde free alcohol (U. S. Industrial Chemicals) and adding 30 cc. of concentrated hydrochloric acid. This solution may be kept in the refrigerator for several weeks.

Procedure: The sample to be tested was hydrolyzed for two hours in 1 N mineral acid on a boiling water bath, using an air reflux condenser to prevent evaporation. It was then cooled in an ice bath, and very carefully neutralized to the phenolphthalein endpoint with 1 N sodium hydroxide added from a burette with a capillary tip and stirred carefully to prevent local over-heating. The pink of phenolphthalein was just removed with a capillary drop of 0.1 N hydrochloric acid. Extreme care had to be taken to avoid local excesses of alkali during neutralization or low results were obtained.

An appropriate aliquot was placed in a graduated tube and diluted to 4 cc. One cc. of freshly prepared acetyl-acetone solution was carefully added. The solutions were mixed and heated on a boiling water bath (with sufficient water to cover the solution) for fifteen minutes.

After cooling, the contents were diluted with 3 cc. of aldehyde-free alcohol and 1 cc. of Ehrlich's reagent was added with stirring. The mixture was then diluted to 10 cc. with alcohol and placed in a water bath at 37° C. for thirty

minutes. The amount of color was then immediately determined by reading in a Coleman colorimeter at 540 mμ.

A blank determination on 4 cc. of distilled water and a series of appropriate standards containing between 25 - 125 μg. of amino sugars were run with each set of determinations.

3. Methyl Pentose Determination. Dische (69) described a group of color reactions of carbohydrates with SH compounds in sulfuric acid which were characteristic for the various classes of carbohydrates. He was able to use one of these reactions for the detection and determination of 2 - 10 μg. of methyl pentose in the presence of a considerable excess of other sugars. The reaction is based upon the breakdown of the sugar by the concentrated sulfuric acid and heat. The combination of this breakdown product with cysteine hydrochloride gives a greenish-yellow color which has characteristic absorption at certain wavelengths. There is a sharp maximum at 400 mμ and practically no absorption at 430 mμ for methyl pentoses. Most of the hexoses have a symmetric absorption curve with a peak at 415 mμ and the absorption at 430 mμ equal to the absorption at 396 mμ. Since methyl pentoses have almost maximum absorption at the latter wavelength, the difference between the extinction coefficients at 396 and 430 mμ is proportional to the amount of methyl pentose present and is not influenced by

considerable excesses of hexoses or pentoses.

If the sugars are heated with the sulfuric acid for three minutes, hexoses form products with cysteine hydrochloride which have characteristic absorption curves with considerable absorption at 430 mμ. When they are heated with the sulfuric acid for ten minutes, hexoses have only a small but definite absorption. Therefore the ten minute heating is more specific for methyl pentoses.

Apparently some methyl pentose is destroyed during hydrolysis, for Dische showed the content was lower on a sample of blood group specific substance which was hydrolyzed first than on an unhydrolyzed sample.

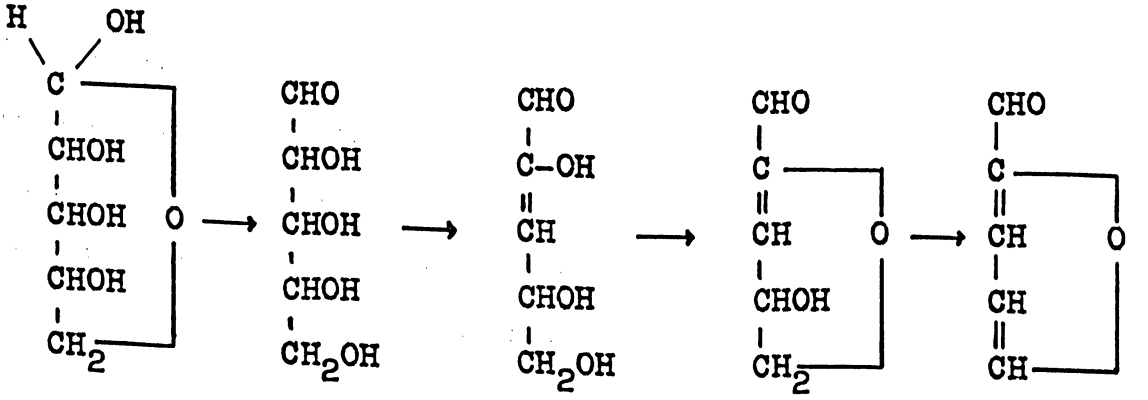
Procedure:

4.5 cc. of sulfuric acid, which had been prepared by adding six volumes of concentrated sulfuric acid carefully to one volume of water, were added carefully to 1 cc. of a sugar solution in a 16 x 150 mm. test tube which was cooled in an ice bath. The mixture was then warmed at 20-25° C., and heated on a boiling water bath for either three or ten minutes exactly. It was then cooled in tap water, and 0.1 cc. of a 3% aqueous cysteine hydrochloride solution was added with shaking. After standing an hour, the mixture was placed in a silica cell and read in a Beckman spectrophotometer, using a tungsten lamp.

A reagent blank and standards of a hexose, such as galactose, and a methyl pentose, such as rhamnose, were run with each set of determinations. A sugar blank was run for each sugar by adding the sulfuric acid but no cysteine in order to determine any absorption due to the action of the sulfuric acid alone. All readings were read against the reagent blank.

The reagent blank and standard galactose readings were taken first to determine at what wave length the density was equivalent to the density at 396 mμ. These variations were apparently due to variations in the time necessary to bring the reaction mixture to 100° C. after immersion in boiling water. The difference in the densities at 396 mμ and at 430 mμ (or the wave length at which the density of galactose was equivalent to its density at 396 mμ) was due to the methyl pentose content.

4. Pentose Determination (70). Pentoses when treated with either concentrated sulfuric or hydrochloric acid form furfural according to the following mechanism:



Hydrochloric acid is less drastic in its action and is the one most commonly used.

The furfural formed couples with phenols, such as orcinol, to give an intensely colored compound which can be used both to detect the presence of pentose and to estimate the amount of pentose quantitatively. Bial made the orcinol reaction more sensitive by carrying out the test in the presence of a little ferric chloride.

Reagents: a. Ferric chloride-hydrochloric acid reagent. A stock solution of 0.5% $FeCl_2$ in concentrated hydrochloric acid was first prepared by dissolving 0.5 grams of $FeCl_3$ in 4.0 cc. of water and making to 100 cc. with concentrated hydrochloric acid. A 0.05% solution was prepared by making a 1:10 dilution of this with concentrated hydrochloric acid.

b. The ferric chloride-orcinol reagent was prepared fresh daily by adding 2 cc. of the 0.05% ferric chloride-hydrochloric acid reagent to 10 mg. of orcinol.

Procedure: An appropriate aliquot of hydrolyzed polysaccharide was diluted to 1 cc. and treated with 2 cc. of the ferric chloride-orcinol reagent. This was carefully mixed with a footed stirring rod and placed in a boiling water bath for thirty minutes. After the tube had cooled for a few minutes in a cold water bath, the stirring rod was washed with water and the mixture diluted to 7.0 cc. After

mixing, the amount of color was read in the Coleman spectrophotometer at 670 mμ.

Because the density deviates from proportionality in the concentration range used, it is necessary to determine a calibration curve. A distilled water blank and a series of standards containing 5-20 μg. of pentose should be run with every set of determinations. Other substances present in the hydrolyzate may also produce color to a slight extent.

5. Uronic Acid Determination. Hexuronic acids, when distilled with hydrochloric acid, decompose into carbon dioxide and a pentose. The pentose, thus formed, yields furfural.

Van der Haar (71) developed a quantitative method for the estimation of uronic acids, based on the fact that each molecule of uronic acid gives off one molecule of carbon dioxide quantitatively on distillation with 12% hydrochloric acid by weight.

The color reaction of the furfural produced from the pentose with naphthoresorcinol was employed by Tollens for the detection of uronic acids. Deichmann (72) modified this to use for quantitative measurements.

Modified Van der Haar Procedure: The apparatus used is shown on the next page.

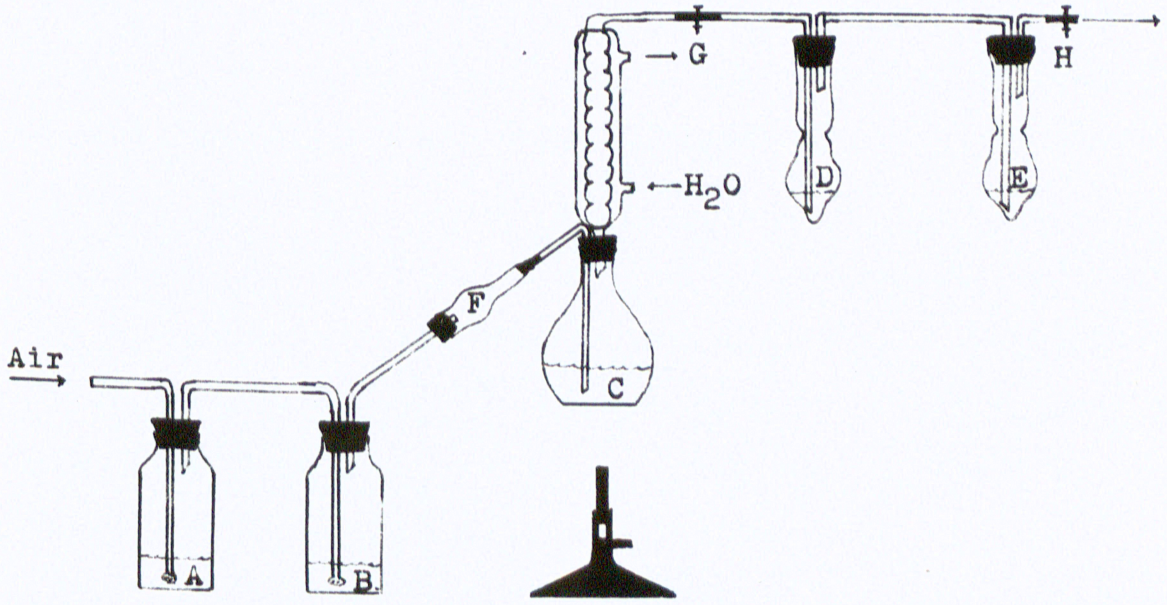


Figure 1. Uronic Acid CO_2 Absorption Train.

A and B. Contain about 100 cc. of 50% KOH to remove CO_2 from the air.
C. Contains sample and 12% HCl.
D and E. Contain standard $Ba(OH)_2$.
F. Cotton to catch vapors.
G and H. Pinch clamps.

The sample to be analyzed and 20 cc. of 12% hydrochloric acid, specific gravity 1.06, were placed in flask C and connected to the rest of the train without any barium hydroxide in flasks D and E. A steady stream of air was pulled through for about fifteen minutes; then clamps H and G were closed in succession. A definite amount of standard $Ba(OH)_2$ was then carefully and quickly placed in flasks D and E. Clamp H was opened slightly, then clamp G, and the stream of air adjusted. Flask C was heated in an oil bath to 133-36° C., and the stream of air gradually increased until boiling started in order to prevent liquid from going the wrong direction in flask C. This was continued for three hours.

The train was then disconnected, the delivery tubes to D and E washed off with CO_2-free water, and the remaining $Ba(OH)_2$ titrated to a phenolphthalein endpoint under CO_2-free conditions.

Blank determinations were run in the same way on the hydrochloric acid alone. The method was standardized with a sample of galacturonic acid.

Modified Deichmann Procedure: The solution containing uronic acid was measured into a 25 cc. graduate with a ground glass stopper. It was diluted to 2.5 cc. with water and 0.1 cc. of 19% hydrochloric acid (43.88 cc. of concentrated

hydrochloric acid were diluted to 100 cc.) was added. The mixture was heated on a 75° C. water bath for firty-five minutes. Then 2.5 cc. of concentrated hydrochloric acid and 0.5 cc. of 10% naphthoresorcinol in 95% ethyl alcohol were added. The mixture was heated on a water bath at 50° C. for ninety minutes, and then cooled. Five cc. of ether were added, and the mixture shaken for about five seconds. After separation of the layers, enough ether was added to make the total ether volume 10 cc. This was mixed by gently rotating the cylinder. The ether layer was removed and read at 570 mμ. Blank determinations were run on distilled water, and a series of standard galacturonic acid determinations were done with each set of determinations.

6. Non-fermentable Reducing Sugar Determination. Somogyi observed that baker's yeast removed glucose quantitatively from a dilute solution in a very short time. Raymond and Blanco (75) found that under the conditions that they employed lactose, galactose, maltose, xylose, arabinose, ribose, and dihydroxyacetone remained quantitatively in the filtrate after the yeast treatment while mannose, fructose, and sucrose were absorbed to the extent of 60-80% depending upon the time of absorption, the concentration of the sugar and the state of the yeast. The difference in the amount of reducing material in the sample before and after fermenta-

tion represents the amount of fermentable reducing substances present.

Reagents: a. Yeast cell suspension was prepared by suspending one cake of Fleischmann's yeast in 80 cc. of water. It was then centrifuged and repeatedly washed until the supernatant was clear and colorless. It was then resuspended in 60 cc. of water. It could be kept for about two weeks in the refrigerator.

b. Reagents for total reducing sugar method, see page 23.

Procedure: In order to test the yeast activity, 1 cc. of the 20% yeast cell suspension was placed in a centrifuge tube, centrifuged, and decanted. Two cc. of glucose standard were added, mixed, and allowed to stand for ten minutes. Then the mixture was centrifuged, and a reducing sugar determination run on one cc. of the supernatant. This should have the same value as the yeast blank because all of the glucose should have been fermented.

The yeast blank was determined by centrifuging 1 cc. of the yeast suspension, decanting, and adding 2 cc. of water to the residue. These were mixed and allowed to stand for ten minutes. The mixture was then centrifuged, and a reducing sugar determination run on a sample of the supernatant.

The test proper was done on 2 cc. of a deproteinized sample of hydrolyzed material such as that described on page 24 and used for the determination of the total reducing sugar content. This sample was added to 1 cc. of the yeast suspension in a centrifuge tube, mixed, and allowed to stand for ten minutes. The mixture was then centrifuged, and sugar determinations run on 1 cc. of the supernatant.

7. Hexose Determination. Dische observed that hexoses react with carbazole in the presence of sulfuric acid and give characteristic colors. Glucose gives a pink color, mannose brown, and galactose an intermediate color. Gurin and Hood (74) found that these colors obeyed Beer's Law and could be quantitatively estimated. Each was found to have a characteristic ratio between the extinction coefficients at 520 and at 420 mμ.

Reagents: a. The sulfuric acid reagent was prepared by adding eight volumes of ice cold sulfuric acid to one volume of cold water. The acid must be of high quality and completely free of nitrate.

b. A 0.5% solution of carbazole in absolute ethyl alcohol was prepared.

Procedure: Nine cc. of the sulfuric acid reagent were accurately measured into a wide test tube and chilled in an ice bath. One cc. of a solution containing between 0.05 and

0.2 mg. of the sample to be analyzed was carefully layered above the acid and chilled for several minutes. After thorough chilling, the tube was slowly agitated without removal from the ice bath in order to avoid any appreciable rise in temperature. Insufficient care in cooling here will cause irregular results in the 420 mμ absorption. After thorough mixing, the tube was removed from the ice bath, and 0.3 cc. of the carbazole reagent was added. The contents were rapidly mixed and heated in a vigorously boiling water bath for ten minutes. The tube was then immersed in an ice-water mixture for several minutes. It was read both at 520 and at 420 mμ. After correcting for reagent blanks, the ratio between the readings at the two wavelengths was calculated.

Glucose, galactose, and mannose standards were run with each set of determinations.

8. Glycogen Determination. Glycogen was determined by the procedure of Good, Kramer, and Somogyi (75). The sample to be tested was dissolved in a concentrated potassium hydroxide solution and the glycogen precipitated from it by adding 1.1-1.2 volumes of 95% alcohol. The separated glycogen was hydrolyzed to free reducing sugar with sulfuric acid, and the reducing sugar determined by the method described on page 23.

Procedure: An appropriate sample of the material to be tested was treated with four times its volume of 25% potassium hydroxide, and heated on a boiling water bath for a few minutes to insure complete solution of the material. The mixture was then diluted to 3 cc. and treated with 3.45 cc. of 95% alcohol. This was thoroughly mixed and then heated on the water bath until it just began to boil. After cooling to room temperature, it was centrifuged and the supernatant decanted. The precipitate was allowed to drain by inverting the tube.

The precipitate was treated with 1 cc. of 1 N sulfuric acid and hydrolyzed on a boiling water bath for four hours. The solution was cooled and made alkaline to phenolphthalein with sodium hydroxide, and 0.5 N sulfuric acid was added until the indicator turned colorless. This was then diluted to a definite volume, filtered, and reducing sugar determinations were run on appropriate aliquots.

9. Determination of the Alcohol-ether Extractable Material. The procedure for the determination of fat in moist feces, as described in Hawk, Oser, and Summerson (76), was used with modifications, because it is fast, convenient and fairly accurate. The free fatty acids and the neutral fat are first extracted with alcohol and ether. Then the soaps of the sample are converted into free fatty acids by the addition of hydrochloric acid, which are then extracted

by shaking with ether and alcohol. The ether is removed and the crude fat purified by means of petroleum ether. The weight of the total fat is obtained. The fat is then dissolved in benzene and titrated with a standardized sodium alcoholate solution, using phenolphthalein as an indicator. The fatty acid is calculated from the titration figures as stearic acid by multiplying the number of cc. of decinormal sodium alcoholate solution required by the factor 28.4. The difference between the weight of total fat and the weight of fatty acids is the weight of neutral fat in the sample extracted.

Procedure: Between 3-5 grams of the material to be extracted were suspended in about 30 cc. of water. This was shaken with 15 cc. of ethyl ether for five minutes. Then 15 cc. of 95% alcohol were added, and the mixture again shaken for five minutes. After the layers had separated, the ether layer was removed and the thin layer of ether which remained diluted with 5 cc. of ether, the vessel slightly agitated, and the ether drawn off. This was repeated five times.

Another 15 cc. portion of ether was added and the mixture shaken for five minutes. After the layers had separated again, the ether was removed and the residue washed as before.

The remaining solution was acidified with 2 cc. of concentrated hydrochloric acid. This was then treated with ether and alcohol as above.

All of the neutral ether extracts were combined, as were all of the acid ether extracts. They were then taken to dryness on a hot water bath and dried over sodium hydroxide under vacuum.

Thirty cc. of petroleum ether, B.P. 30-65° C., were added to each and allowed to stand overnight. The petroleum ether extracts were then filtered and the flasks and filter papers washed five times with 5 cc. of petroleum ether. These solutions were then evaporated in a stream of air and dried in an oven at 100° C. for four hours.

The non-petroleum ether extractable material was washed with a total of 25 cc. of 95% alcohol and 25 cc. of ethyl ether. These extracts were then taken to dryness on a hot water bath, dried under vacuum over sodium hydroxide, and weighed.

The petroleum ether extractable materials were weighed and then dissolved in 50 cc. of benzene. They were then heated almost to the boiling point and titrated with a solution of standardized sodium alcoholate to a phenolphthalein endpoint. The fatty acid, calculated as stearic acid, was calculated by multiplying the number of cc. of 0.1 N sodium alcoholate solution required by the factor 28.4.

10. Determination of Total Phosphorus. The Fiske and Subbarow method (77) was used for the determination of phosphorus. The phosphorus is liberated from combination by ashing with sulfuric acid and nitric acid. The liberated or free phosphorus forms phosphomolybdate with molybdic acid, and this phosphomolybdate is reduced by a sulfonic acid reagent to form a blue color complex which can be determined colorimetrically.

Reagents: a. The neutral molybdate solution consisted of a 2.5% solution of ammonium molybdate.

b. The sulfonic acid reducing reagent was prepared by mixing 142.5 g. $NaHSO_3$ (Merck), 10.0 g. Na_2SO_3 (anhydrous), and 2.5 g. 1-amino-2-naphthol-4-sulfonic acid. 15 g. of this mixture were dissolved in 100 cc. of water and filtered if necessary before using.

Procedure: A sample of the material to be tested, containing about 30 µg. of phosphorus, was heated with 3 cc. of 5 N sulfuric acid, using two glass beads to prevent bumping, until dense white SO_3 fumes started to form. Two drops of concentrated nitric acid were carefully added and the mixture heated until the brown fumes disappeared and the dense white fumes appeared again. After cooling, 2 cc. of a 2% urea solution were carefully added in order to remove the oxides of nitrogen that had formed. The mixture was boiled

for about one minute, then cooled to room temperature. It was then diluted to about 20 cc. Three cc. of the neutral molybdate reagent were added, and then 1.2 cc. of the sulfonic acid reducing agent. The mixture was diluted to 30 cc. and allowed to stand at room temperature for thirty-five minutes. It was then read in a Coleman spectrophotometer at 660 mμ against distilled water. A blank determination was done on distilled water, and an appropriate series of standards were run at the same time.

11. Determination of Acid-soluble Phosphorus. The acid-soluble phosphorus is that which is not precipitated when the sample to be tested is treated with a solution of trichloroacetic acid. It represents that phosphorus which is not bound to protein.

Procedure: The sample to be tested was treated with an equal volume of 20% trichloroacetic acid and centrifuged. The supernatant was treated with one-tenth its volume of 4 N sulfuric acid and heated on a boiling water bath for two hours to decompose the trichloroacetic acid. An appropriate sample of this was digested according to the procedure for total phosphorus described above.

12. Determination of Total Nitrogen by a Modified Micro-Kjeldahl Method (78). This method for the determination of nitrogen in organic substances consists in converting the nitrogen into ammonium acid sulfate while decomposing

the organic matter with boiling sulfuric acid. Usually the decomposition is hastened by adding a salt, such as sodium sulfate, to raise the boiling point of the mixture and a catalyst, such as selenium or mercury. After the organic matter has been decomposed completely, the solution is made alkaline with sodium hydroxide which liberates the ammonia. The ammonia is distilled, usually with steam, into a measured volume of standard acid solution. The excess of the standardized acid is then titrated with standard sodium hydroxide, using methyl red as an indicator.

Reagents: The mercury catalyst was prepared by dissolving 10 g. of red mercuric oxide in 12 cc. of concentrated sulfuric acid which had been diluted to 100 cc. with water.

Procedure: For the digestion, 0.5 g. of sodium sulfate (Merck anhydrous) was placed in a small Kjeldahl flask, and the nitrogen sample, containing about 500 μg. of nitrogen, was added to it. 0.5 cc. of mercury catalyst solution and 2 cc. of 18 N sulfuric acid were added, and the mixture was heated slowly to remove the water. It was then digested until clear, and then for another thirty minutes to insure complete digestion. After cooling about two minutes, 3 cc. of water were carefully used to wash down the sides of the flask.

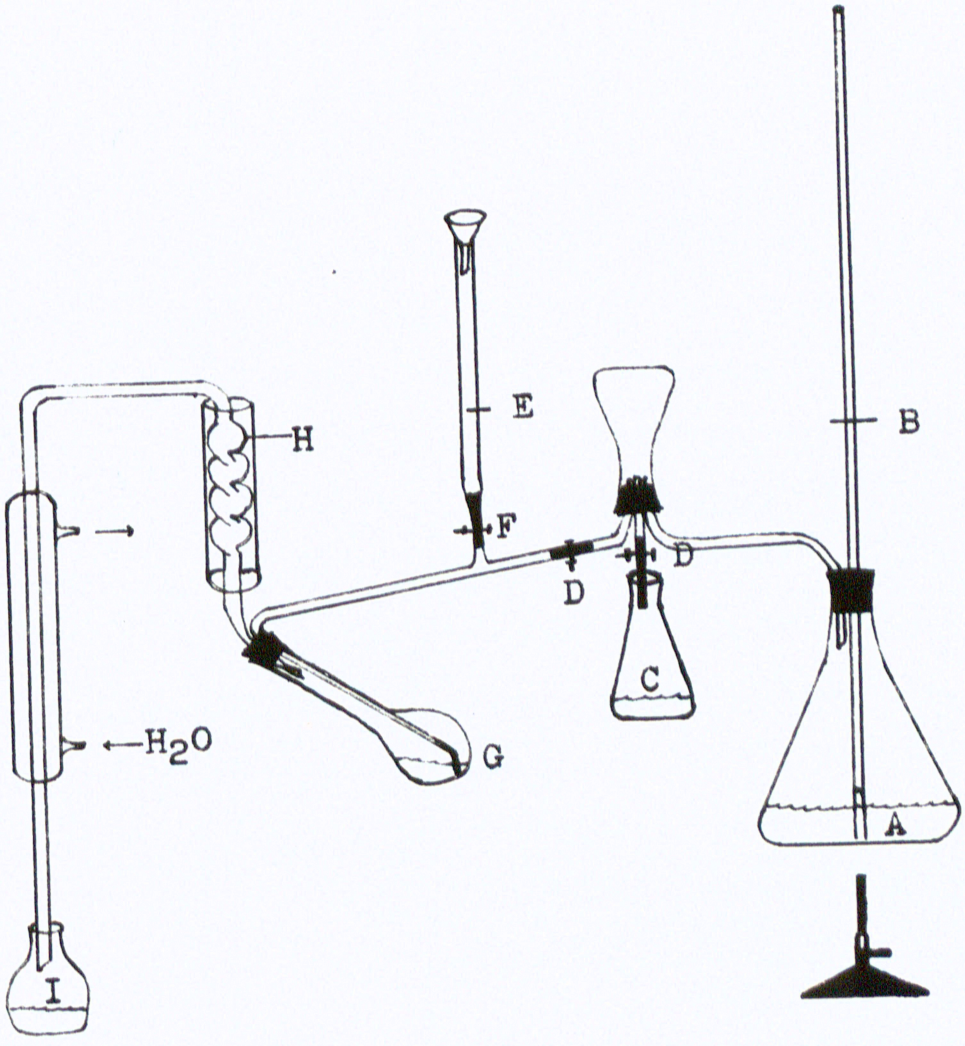


Figure 2. Micro-Kjeldahl Apparatus.

A. Steam generator.
B. Steam Safety valve.
C. Trap for waste steam.
D. Pinch clamps to control steam.
E. Sodium hydroxide.
F. Pinch clamp to control sodium hydroxide.
G. Digestion mixture.
H. Kjeldahl trap and shield
I. Standard acid.

The distillation apparatus was carefully steamed out, and a receiving flask containing a known volume of 0.01 N hydrochloric acid and three drops of methyl red indicator placed in position with the delivery tip below the surface of the acid. A drop of methyl red indicator and about 0.2 g. of zinc dust, to reduce the mercuric oxide to metallic mercury, were added to the digested material. The Kjeldahl flask was connected to the distillation apparatus, and 10 cc. of approximately 10 N sodium hydroxide were slowly added. Steam was allowed to enter, and the distillation continued for ten minutes. The delivery tip was raised to about one-half inch above the acid and the steaming continued for one more minute.

The remaining amount of hydrochloric acid in the receiving vessel was titrated with 0.01 N sodium hydroxide under carbon dioxide free conditions to the methyl red endpoint.

Reagent blanks and an appropriate series of a standard ammonium sulfate solution were determined each day.

13. Determination of Total Nitrogen by the Nessler Method (79). The sample to be analyzed is digested with concentrated sulfuric acid. A little saturated sodium sulfate is added to raise the boiling point and thus hasten the

digestion. A small amount of a substance like sucrose will also help in making the digestion more complete. Hydrogen peroxide is used to complete the oxidation. The ammonia is determined by the color produced when Nessler's reagent, an alkaline potassium mercuric iodide solution, is added. The colored product which results from the reaction is colloidal in nature and flocculates on long standing. If the solution contains too much ammonia a distinct orange precipitate is formed. More dilute solutions form a yellow-brown solution. This color may be read colorimetrically.

Reagents: Nessler's reagent was prepared from a commercial Nessler's compound prepared by Paragon C. and C. Company, Inc., New York 58, N. Y. 100 g. of sodium hydroxide were dissolved in a liter of water. 850 cc. of this were placed in a liter volumetric flask. One bottle of the Nessler's compound, 68 g., was dissolved in 100 cc. of water and added to the sodium hydroxide. The mixture was then diluted to a liter and stored in a flask protected with a soda lime tube. It was allowed to stand at least 48 hours before using.

Procedure: The sample, containing about 100 µg. of nitrogen, was placed in a Nessler tube calibrated at 35 and 50 cc. Two cc. of 10 N sulfuric acid, 0.4 cc. of saturated sodium sulfate, a trace of Bureau of Standards sucrose, and two

small glass beads were added. The mixture was then carefully digested until all charring was completed. A few drops of hydrogen peroxide were then added with caution, and the mixture again boiled for a minute. After cooling, it was diluted to 35 cc., and care was taken to see that the sulfuric acid mixture and water were thoroughly mixed. Fifteen cc. of the Nessler's reagent were rapidly blown in from a special wide tipped pipette, and the tube immediately stoppered and mixed thoroughly. The color density was then read at 440 mμ in a Coleman spectrophotometer.

The total volume was at times decreased to 10 cc. by using a sample containing about 20 μg. of nitrogen, adding 0.4 cc. of 10 N sulfuric acid and 0.1 cc. of saturated sodium sulfate, diluting to 7.0 cc., and adding 3 cc. of Nessler's reagent as described above.

Blanks of the reagents and appropriate standards of an ammonium sulfate solution were analyzed with each series of determinations.

14. Determination of Non-protein Nitrogen. A protein-free solution can be prepared by precipitating any protein present with a solution of trichloroacetic acid. Any nitrogen which is not combined with the protein will remain in the supernatant and can be determined by any standard method of nitrogen determination, such as the Kjeldahl or Nessler methods described on page 43 and page 46.

Procedure: A sample of a suspension or solution of the material to be tested was treated with twice its volume of 20% trichloroacetic acid solution, diluted to a definite volume with water, and centrifuged. Appropriate samples of the supernatant were then analyzed for nitrogen.

15. Determination of Purine Nitrogen. The procedure of Graff and Maculla (80) was used to prepare the hydrolysis mixture. Usually the sample is refluxed with hydrochloric acid, a procedure which is often very lengthy, in order to get complete hydrolysis of tissue. This often causes large losses of purine. The modified procedure employs formic acid, which is a very effective protein solvent. A mixture of 10 N formic acid and 1 N hydrochloric acid yields a clear, almost melanin-free solution, has a hydrogen ion activity equivalent to that of 4 N hydrochloric acid, does not form the inconvenient viscous solution which 10 N formic acid alone forms with protein, and reduces the losses resulting from decomposition of the purine during refluxing.

After neutralization of the hydrolysis mixture, a modification of the procedures of Hitchings (81) and Hitchings and Fiske (82) was used for the actual purine determination. It employs the fact that all purines are precipitated by copper sulfate and sodium bisulfite in boiling solutions as a purine-copper-bisulfite complex. Some

non-purine nitrogen is sometimes carried down in the first copper-bisulfite precipitation, so a reprecipitation is usually performed, which apparently removes any extraneous nitrogen completely. The use of hydrogen sulfide to decompose the complex is more satisfactory than the use of sodium sulfide which does not give complete recovery of the purines and introduces large amounts of electrolyte, which interfere with later steps. The hydrogen sulfide can be easily removed by evaporation. A slight loss of purine may occur due to the fact that purines are absorbed on heavy metal sulfides.

The nitrogen of the final solution is determined by the micro-Kjeldahl method, page 43.

Procedure: Samples of the meconium suspension were refluxed for one hour with five times their volume of 1 N hydrochloric acid in 10 N formic acid. These mixtures were then cooled and neutralized with 10 N sodium hydroxide to the phenolphthalein endpoint. After heating on a boiling water bath, the purine bases were precipitated by adding 0.8 cc. of a saturated solution of sodium bisulfite and 1.0 cc. of a 10% $CuSO_4 \cdot 5H_2O$ solution. After heating for three minutes, the precipitate was collected in sintered glass crucibles, washed with 20 cc. of water, and then carefully suspended in 3 cc. of 3 N hydrochloric acid and about 15 cc. of water. The suspension was heated on a hot plate for about twenty

minutes, then treated with hydrogen sulfide for three minutes, diluted to volume, and filtered.

Aliquots were evaporated to dryness and then dissolved in 8 cc. of water. These solutions were then heated on a boiling water bath and the purine bases reprecipitated by the addition of 1 cc. of a 10% $CuSO_4 \cdot 5H_2O$ solution and 0.8 cc. of a saturated solution of sodium bisulfite. After heating for three more minutes, the precipitates were collected by centrifugation and washed twice with hot water. The precipitates were boiled with 2 cc. of 3 N hydrochloric acid, diluted to 10 cc. with hot water and treated for three minutes with hydrogen sulfide. The solutions were diluted to volume and filtered. Aliquots of the supernatants were then analyzed for nitrogen.

16. Acetyl Determination. The colorimetric micro-analytical method for the determination of acetate that was described by Hutchens and Kass (83) did not give reliable results for us. The method employed the formation of a basic lanthanum acetate which absorbs iodine, to give a characteristic blue color which could be determined colorimetrically. Inorganic ions such as chloride, phosphate, sulfate, calcium, and magnesium interfere and have to be removed. In our hands the speed of addition of the reagents, the order of addition, the length of heating, and the amount

of carbonate present in the solutions were some of the factors which caused great variations in the results. Because of the great number of existing variables, the acetyl content was determined by distillation.

The method for the determination of acetyl groups as described in Kabat and Mayer (84) was found to be the most satisfactory. The sample to be analyzed is hydrolyzed with p-toluene sulfonic acid, thus freeing any combined acetyl groups. This mixture is then steam distilled, and the volatile acids in the distillate titrated with standard sodium hydroxide, and calculated as acetyl groups.

Reagents: The p-toluene sulfonic acid used was impure and gave very high blanks so it was made useable as follows: 20 cc. of a 25% solution of p-toluene sulfonic acid were distilled under vacuum to dryness, and this was repeated three more times. The dry acid was then redissolved in 20 cc. of water.

Procedure: The sample to be tested was placed in a small Kjeldahl flask and treated with 2 cc. of 25% p-toluene sulfonic acid. The mixture was heated on a boiling saturated salt bath for two hours, under an adequate reflux condenser. After cooling and rinsing of the condenser, the flask was placed in the micro-Kjeldahl apparatus, see page 43, and steam distilled. Approximately 30 cc. fractions

were collected until they reached a constant blank value (usually four or five fractions).

The fractions were then titrated with a standard sodium hydroxide solution.

B. Serological Methods (25).

1. Determination of Blood Type. Samples of the baby's blood were collected by pricking their heels and of the mother's blood by pricking their fingers. The blood was caught in a small tube and allowed to clot. It was then carefully defibrinated.

a. Types A, B, AB, and O. Two small drops of defibrinated blood were placed on a microscope slide. A drop of undiluted anti-A typing serum about the same size as the drop of blood was placed near one and a drop of undiluted anti-B typing serum (human sera supplied by the American Hospital Supply Corporation) near the other. The sera were then carefully mixed with the blood and observed for signs of agglutination. Usually agglutination occurred immediately. The blood type was then determined according to the following chart:

Blood Type	Agglutination with Anti-A	Anti-B
A	+	-
B	-	+
AB	+	+
O	-	-

b. Type Rh. For the determination of Rh, one drop of defibrinated blood was mixed carefully with one drop of human anti-Rh_0 (anti-D) serum for slide testing (supplied by the American Hospital Supply Corporation) and observed for agglutination.

c. Types M and N. For the determination of M or N type, one drop of defibrinated blood was mixed with two large drops of 0.85% saline and a little anti-M or anti-N powdered serum (supplied by Lederle Laboratories) and observed for agglutination.

d. Type O with Human Anti-O Serum. A sample of human anti-O serum from Mary Hartnett was obtained from the Blood Grouping Laboratory, 300 Longwood Avenue, Boston 15, Massachusetts. To get positive agglutination with type O blood it was necessary to do the tests in the cold room at 38° F., since agglutination is much more sensitive at low temperatures.

2. Standardization of the Typing Sera. The serum to be used was standardized each time a new bottle was opened so that the results could be quantitated.

a. Anti-A and Anti-B Sera. 0.25 cc. of anti-A serum was added to 0.75 cc. of 0.85% saline to give a 1:4 dilution. 0.5 cc. of this was added to 0.5 cc. of saline to give a 1:8 dilution. 0.5 cc. of this serum dilution was again added to 0.5 cc. of saline, and this repeated until a dilu-

tion of 1:2048 was reached.

A sample of blood from a person of blood type A, who could supply a sample of blood each time the serum was used, was defibrinated or heparinized and suspended in 15 cc. of saline. The red cells were then separated by rapid centrifugation and resuspended in sufficient 0.85% saline to make a 0.5% suspension.

0.1 cc. of each serum dilution was treated with 0.1 cc. of 0.85% saline and 0.1 cc. of the red cell suspension. It was then allowed to stand at room temperature for two hours.

As one becomes experienced, he will become familiar with various indications of the amount of agglutination. The following is the most reliable method for determining the agglutination.

The tests were carried out in small serological tubes. After standing for the required two hours, they were centrifuged lightly. Each tube was then carefully picked up in the left hand and tapped with the forefinger of the right hand. The red cells in the bottom of the tube were carefully observed for their characteristic behavior. The suspension was then poured carefully onto a microscope slide and examined under low power on a microscope.

The amount of agglutination was recorded as follows:

++++ Macroscopically it separated from the bottom of the tube as one or two large clumps of cells which were broken only on quite vigorous agitation.

+++ Macroscopically it separated from the bottom of the tube in several large clumps of cells which would break quite easily upon agitation.

++ Macroscopically numerous small clumps of cells could be distinguished. If not carefully handled, these clumps would break. Microscopically these clumps appeared quite large, and there were very few free cells.

+ Macroscopically the clumps would appear to swirl up from the bottom. If held toward a light, the agglutination might be seen, but it was fairly difficult. Microscopically this appeared as numerous small clumps with quite a few single cells.

± This would appear to swirl up from the bottom when the tube was agitated. No indication of agglutination could be seen macroscopically. Upon microscopic examination only one or two small clumps of agglutinated cells could be seen. The rest of the cells were all free.

- This appeared to swirl up from the bottom when agitated. Microscopically no agglutination could be seen.

A typical anti-A serum standardization is shown below:

Dilution	1:4	1:8	1:16	1:32	1:64	1:128
Agglutination	++++	++++	++++	++++	++++ Max.	+++

Dilution	1:256	1:512	1:1024	1:2048
Agglutination	++	+	-	-

The anti-B serum was standardized in a similar manner, using type B blood.

For most determinations a 1:8 dilution of anti-A or anti-B serum was used. This contained three or four times as much serum as needed to give maximum agglutination.

b. Anti-O Serum. 0.25 cc. of the serum was added to 0.75 cc. of albumin (Armour Laboratories albumin obtained from bovine plasma, prepared for use in Rh testing, and containing 0.3 g. per cc.) to give a 1:4 dilution. 0.5 cc. of this was added to 0.5 cc. of albumin to give a 1:8 dilution. 0.5 cc. of this serum dilution was again added to 0.5 cc. of the albumin, and this repeated until a dilution of 1:2048 was reached.

A sample of blood from a person of blood type O, who could supply a sample of blood each time the serum was used, was defibrinated and suspended in 15 cc. of 0.85% saline. The red cells were then separated by rapid centrifugation and resuspended in sufficient 0.85% saline to make a 1.0% suspension. This suspension was mixed with an equal volume of albumin, to make a 0.5% cell suspension.

0.1 cc. of each serum dilution was treated with 0.1 cc. of 0.85% saline and 0.1 cc. of the red cell suspension, and allowed to stand in the cold room at 38° F. for two hours. The tubes were then centrifuged in the cold, and the amount of agglutination read in the same way as for A or B activity except that extreme care had to be taken or the agglutination would be broken by too vigorous agitation. Even with concentrated serum a +++ agglutination was the greatest observed.

c. Anti-Rh_o Serum. 0.2 cc. of serum for tube testing (supplied by the Ortho Pharmaceutical Corporation) was mixed with 0.2 cc. of saline, and this was repeated until a dilution of at least 1:512 was obtained.

A sample of blood from a person who had type O, Rh_o^+ blood and could supply a sample of blood each time the serum was used, was defibrinated or heparinized and suspended in 15 cc. of 0.85% saline. The red cells were then separated by rapid centrifugation and resuspended in sufficient 0.85% saline to make a 2% suspension.

0.1 cc. of each serum dilution was treated with 0.1 cc. of saline and 0.1 cc. of the blood suspension. These were incubated at 37° C. for one hour. The amount of agglutination was read as usual except that extreme care had to be exercised to avoid breaking of the agglutination.

d. Anti-M and Anti-N Sera. 0.01 g. of anti-M or anti-N

was diluted with 0.4 cc. of 0.85% saline and a series of 1:4 dilutions with saline were prepared.

A sample of blood from a person with MN type blood, who could supply a sample of blood each time the serum was used, was defibrinated and suspended in 15 cc. of 0.85% saline. The red cells were then separated by rapid centrifugation and resuspended in sufficient 0.85% saline to make a 0.5% suspension.

To 0.1 cc. of each dilution, 0.1 cc. of saline, and 0.1 cc. of the cell suspension were added. These were mixed and allowed to stand at room temperature for two hours. The amount of agglutination was read as usual.

3. Determination of Blood Group Activity (56). Substances which possess the same activity as the agglutinogens which are present in the blood cells are also found in many body fluids and tissues. If solutions or suspensions of these materials are allowed to stand in contact with a specific serum for a while they will combine with the agglutinins in the serum. When a suspension of red cells of the type for which the serum is specific is then added, there are no agglutinins left to combine with the specific substance the cells contain. The absence of agglutination in these cells can be used to show the presence of active materials.

a. Determination in Saliva. Saliva specimens were obtained from the babies by swabbing their mouths with sterile cotton swabs. A sample of saliva was obtained from the mothers. As soon as possible, 1 cc. of 0.85% saline was added to each sample, and the mixtures were heated on a boiling water bath for five minutes to inactivate any enzymes which might destroy the blood-group activity. The tubes were stoppered and kept in the deep-freeze until they could be tested.

To test for the presence of type A blood group activity, 0.1 cc. of the saliva-saline solution was treated with 0.1 cc. of a 1:8 dilution of anti-A typing serum. After standing for an hour at room temperature, this mixture was treated with 0.1 cc. of freshly prepared 0.5% blood type A red cell suspension. This was allowed to stand for two hours at room temperature. The tubes were then centrifuged and the amount of agglutination read.

The presence of type B blood group activity was tested for in the same way using anti-B serum and type B red cells.

To test for the presence of type O blood group activity, 0.1 cc. of the saliva-saline solution was treated with 0.1 cc. of the serum dilution, which had been prepared by diluting 0.1 cc. of the serum with 0.7 cc. of albumin. After standing in the cold room at 38° F. for an hour, 0.1 cc. of freshly prepared 0.5% blood type O red cell suspension, prepared as

described on page 57, was added. The mixture was then mixed and allowed to stand in the cold for two hours. The tubes were then centrifuged in the cold and the amount of agglutination read.

b. Determination in Meconium and Amniotic Fluid. A suspension of one part meconium to three parts of water was prepared with the aid of a Waring blendor. These suspensions and amniotic fluid were centrifuged slightly to remove insoluble material. If large amounts of blood group activity were suspected, a series of 1:10 dilutions with 0.85% saline were prepared.

A, B, and O blood group activity was tested for in the same was as described above for testing saliva. M and N blood group activity was tested for in the same way as A and B activity except that a 1:60 dilution of anti-M and a 1:30 dilution of anti-N sera and MN red cells were used.

Rh_o blood group activity was tested for as follows: 0.1 cc. of the suspension of material to be tested was treated with 0.1 cc. of the serum dilution and incubated at 37° C. for an hour. 0.1 cc. of a 2% fresh red blood cell suspension was then added and the mixture again incubated at 37° C. for an hour. It was then centrifuged and the amount of agglutination determined.

The following precautions should be observed: (1) The measuring should be done as carefully as possible with

bacteriological pipettes, calibrated to 0.01 cc. (2) The solution to be tested should be homogeneous and should not contain any substances, such as alcohol or a high salt concentration, which would hemolyze the blood cells. (3) The red blood cells from the same person should be used for each test as were used for the standardizations. (4) The red cells should be fresh every day. They become less sensitive on standing, even in the cold. (5) The amount of centrifugation should only be sufficient to collect the cells and not enough to pack them too firmly. (6) Care must be taken to avoid too violent agitation of the cells when reading. (7) Care must be taken to avoid reading agglutination falsely due to rouleaux formation when a suspension of cells is allowed to stand under certain conditions. Also false agglutination may occur around foreign particles.

III. Comparison of Normal Meconium and Meconium Ileus

A. Aim of the Investigation.

Normal meconium is a sticky, mucus-like excretion of newborn babies. The material usually has a very dark green color, but a few specimens from apparently normal children are obtained which have a greenish-brown to brown color. Suspensions of normal meconium in water often are very viscous, but a few have very little viscosity. Meconium is largely soluble in water, yielding only insignificant amounts of insoluble material upon centrifugation. Most of the insoluble material consists of lanugo hairs.

In contrast to normal meconium, meconium ileus has a putty-like consistency - a characteristic which prevents its ready excretion by the infant. From a few samples of meconium ileus that are available it would seem that it is present in much larger amounts, than meconium in a normal baby.

Because of the sharp contrast in consistency between normal meconium and meconium ileus, it was of interest to characterize the chief chemical components of each and to determine the gross chemical differences between them.

B. Materials Used for the Investigation.

The normal meconium used was a pool of the meconium from three normal babies, which was kept in the deep freeze until needed.

The meconium ileus was from Infant Zinser, obtained on autopsy February 14, 1949 and preserved by keeping in the deep freeze until used.

The crude meconium was used for the determination of dry weight and ash content, but a homogeneous suspension of meconium was used for the other determinations. The suspensions were prepared by mixing about five grams of the meconium with forty cc. of water in a Waring blendor.

C. Determination of the Moisture Content.

A sample of the meconium was weighed onto a piece of folded, ashless filter paper in a vessel which had been carefully brought to constant weight by heating at 110° C., cooling in a desiccator, and weighing. The vessel and sample were then dried at 110° C. and brought to constant weight.

Results:

Normal meconium	Average dry weight	27.6%
	Average moisture content	72.4%
Meconium ileus	Average dry weight	29.8%
	Average moisture content	70.2%

Discussion: Meconium had a moisture content very similar to that of ordinary tissue, and disproved the statement of some workers (23) that meconium contained little water.

D. Determination of the Ash Content.

Platinum crucibles were brought to constant weight. The sample of meconium on the filter paper from the determination of dry weight was carefully ashed and brought to constant weight.

Results:

Normal meconium	Average Ash	1.1% on wet weight basis 4.1% on dry weight basis
Meconium ileus	Average Ash	0.54% on wet weight basis 1.8 % on dry weight basis

Discussion: The greatest part of both meconium and meconium ileus consisted of organic matter, the ash content being only minimal.

E. Determination of the Insoluble Material.

The meconium suspensions were quite homogeneous but did yield small amounts of insoluble material when heavily centrifuged, so it was of interest to determine the amount of this insoluble material accurately.

Five cc. of the meconium suspensions were heavily centrifuged. The insoluble material was separated and washed four

times with 5 cc. of distilled water, three times with 5 cc. of a 5% saline solution, three times with distilled water, twice with 95% alcohol, and three times with anhydrous ether. The remaining insoluble material was then dried in an oven at 100° C. and weighed.

Results:

Normal meconium	0.5% insoluble material on wet weight basis 1.9% insoluble material on dry weight basis
Meconium ileus	2.7% insoluble material on wet weight basis 9.0% insoluble material on dry weight basis

Discussion: A large amount of the insoluble material appeared to consist of lanugo hair.

F. Determination of the Total Nitrogen Content.

The total nitrogen content was determined by the micro-Kjeldahl method described on page 43.

Results:

Normal meconium	1.9% nitrogen on wet weight basis 6.9% nitrogen on dry weight basis
Meconium ileus	4.3% nitrogen on wet weight basis 14.4% nitrogen on dry weight basis

Discussion: These values showed that the two types of meconium differed greatly in the amount of total nitrogen calculated on the basis of dry weight.

G. Determination of Non-protein Nitrogen.

The non-protein nitrogen was determined by the method described on page 43, using the micro-Kjeldahl method to determine the nitrogen.

Results:

Normal meconium	84.1% of the nitrogen was non-protein
Meconium ileus	5.4% of the nitrogen was non-protein

Discussion: These results showed that normal meconium contained very little protein, while meconium ileus contained very large amounts of protein. By calculation, it was seen that 0.3% of normal meconium on the wet weight basis or 1.1% on the dry weight basis was protein nitrogen, and 4.0% of meconium ileus on the wet weight basis or 13.6% on the dry weight basis was protein nitrogen. When the amount of nitrogen was multiplied by the protein factor, 6.25*, it was seen that normal meconium contained about 1.9% protein and meconium ileus about 25% protein, calculated on the wet weight basis. On the dry weight basis, normal meconium contained about 6.9% protein and meconium ileus about 85% protein.

H. Determination of Purine Nitrogen.

The purine nitrogen was determined by the method

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* 6.25 is the usual factor employed for the calculation of protein from the nitrogen content and is based on the assumption that proteins contain on the average 16% nitrogen.

described on page 49.

Results:

Normal meconium 1.4% of total nitrogen was purine nitrogen

Meconium ileus 2.9% of total nitrogen was purine nitrogen

Discussion: The results were so low that they were almost within experimental limits. Attempts were made to test for non-protein purine, but the results were too low to be significant at all. These results definitely showed that meconium did not consist of nucleic acid residues accumulated during embryonic development.

I. Determination of the Alcohol-ether Extractable Material.

The alcohol-ether extractable material was determined by the method described on page 39.

Results:

Normal Meconium

3.4% alcohol-ether extractable on wet weight basis
12.2% alcohol-ether extractable on dry weight basis
1.8% petroleum ether soluble on wet weight basis
6.5% petroleum ether soluble on dry weight basis
0.2% stearic acid on wet weight basis
0.9% stearic acid on dry weight basis

Meconium ileus

2.9% alcohol-ether extractable on wet weight basis
9.7% alcohol-ether extractable on dry weight basis
1.3% petroleum ether soluble on wet weight basis
4.2% petroleum ether soluble on dry weight basis
0.3% stearic acid on wet weight basis
0.9% stearic acid on dry weight basis

Discussion: From these results it would seem that meconium ileus was not formed as a result of a primary deficiency in lipase activity in the intestinal tract as some workers (21) have believed.

J. Determination of Reducing Sugar.

The reducing sugar content was determined by the method described on page 23.

Results:

Normal meconium

9.8% total reducing sugar on wet weight basis
35.5% total reducing sugar on dry weight basis
0.3% free reducing sugar on wet weight basis
1.1% free reducing sugar on dry weight basis
3.1% of total reducing sugar was free

Meconium ileus

2.6% total reducing sugar on wet weight basis
8.7% total reducing sugar on dry weight basis
0.1% free reducing sugar on wet weight basis
0.2% free reducing sugar on dry weight basis
2.7% of total reducing sugar was free

Discussion: A large percentage of the normal meconium consisted of reducing sugar calculated as glucose. Since most of the other reducing sugars have a lower reducing value than glucose, the actual percentage was probably considerably higher than the above figures showed. This amount of reducing sugar also accounted for a considerable portion of the meconium ileus, not accounted for by the protein content.

Probably the reducing sugar existed in the form of a complex polysaccharide, since there was very little free reducing sugar.

K. Determination of Total Phosphorus

The total phosphorus content was determined by the method described on page 42.

Results:

Normal meconium	0.01% phosphorus on wet weight basis 0.04% phosphorus on dry weight basis
Meconium ileus	0.02% phosphorus on wet weight basis 0.07% phosphorus on dry weight basis

L. Determination of Acid-soluble Phosphorus.

The acid-soluble phosphorus was determined by the method described on page 48.

Results:

Normal meconium	57% of the phosphorus was acid-soluble
Meconium ileus	9% of the phosphorus was acid-soluble

Discussion: The phosphorus content of meconium was only minimal. Most of the phosphorus of normal meconium was not bound to protein while almost all of the phosphorus in meconium ileus was bound to protein or material which was precipitated by trichloroacetic acid.

M. Determination of Glycogen.

The glycogen content was determined by the method described on page 38.

Results:

Normal meconium	0.08% glycogen on wet weight basis 0.3 % glycogen on dry weight basis
Meconium ileus	0.03% glycogen on wet weight basis 0.1 % glycogen on dry weight basis

Discussion: The meconium contained only minor traces of glycogen, calculated as glucose.

N. Determination of Non-fermentable Reducing Sugar.

The non-fermentable reducing sugar content was determined by the method described on page 35.

Results:

Normal meconium	5.2 mg. reducing sugar in 0.5 cc. suspension 5.1 mg. non-fermentable reducing sugar in 0.5 cc. suspension
Meconium ileus	1.5 mg. reducing sugar in 0.5 cc. suspension 1.4 mg. non-fermentable reducing sugar in 0.5 cc. suspension

Discussion: Most of the reducing sugar that was present in meconium and meconium ileus was not glucose but some sugar which was non-fermentable, such as galactose.

O. Conclusion

The major components of normal meconium and meconium ileus were determined. The results are summarized in Tables I and II. No attempt was made to determine the inorganic content of such constituents as sodium, iron, and sulfate; the bile pigments; cholesterol; and other constituents which have been reported to be present in meconium or might possibly be present, in minimal amounts.

Normal meconium was found to contain little protein and large amounts of carbohydrates probably in the form of a polysaccharide-amino acid complex. The low purine and phosphorus contents made the theory that meconium formed as a result of the accumulation of nuclear residues during fetal development untenable.

Meconium ileus consisted largely of protein that appeared bound with small amounts of carbohydrate. These findings would tend to confirm the theory that one of the main causes for the formation of meconium ileus is the lack of tryptic activity in the intestines.

Table I

COMPARISON OF NORMAL MECONIUM WITH MECONIUM ILEUS

Sample	On wet weight basis						
	Moisture	Ash	Alcohol-Ether Extd.	Reducing Sugar	P	Protein	Total
	%	%	%	%	%	%	%
Normal Meconium	72.4	1.1	3.4	9.8	0.01	1.9	89
Meconium Ileus	70.2	0.54	2.9	2.6	0.02	25.0	101

Table II

COMPARISON OF NORMAL MECONIUM WITH MECONIUM ILEUS

Sample	Dry Weight	On dry weight basis: Ash	Total Nitrogen	Alcohol-Ether Extd.	Total Reducing Sugar	Total Phosphorus	Non-protein Nitrogen	Purine Nitrogen	Free Reducing Sugar	Glycogen	Acid-soluble Phosphorus
	%	%	%	%	%	%	% of total N	% of total N	% of total sugar	% of total sugar	% of total P
Normal Meconium	27.6	4.1	6.9	12.2	35.5	0.04	84.1	1.4	3.1	1.0	57
Meconium Ileus	29.8	1.8	14.4	9.7	8.7	0.07	5.4	2.9	2.7	1.0	9

IV. Purification and Fractionation of Meconium

A. Aim of the investigation.

From the chemical data obtained in the comparison of normal meconium with meconium ileus, it was seen that a major portion of normal meconium probably consisted of a polysaccharide and of nitrogen compounds that were non-protein in character. In order to determine more fully the form of these various components, several different methods of purification and fractionation were attempted, and the products from these carefully studied and analyzed.

Meconium ileus was also studied in an effort to characterize the protein portion in addition to the polysaccharide that it contained.

B. Preliminary Study of Normal Meconium.

A suspension of the mixed meconium from three normal babies was tested to see if the supernatant after treatment with certain deproteinizing agents still contained hydrolyzable carbohydrate.

One sample was treated with the Somogyi deproteinizing agents, barium hydroxide and zinc sulfate, see page 23, and an aliquot of the supernatant hydrolyzed with 1 N sulfuric acid for four hours.

Another sample was treated with a solution of $Pb(OAc)_2$. The supernatant was treated with hydrogen sulfide. The lead

sulfide was centrifuged off, and an aliquot of the supernatant hydrolyzed with 1 N sulfuric acid for four hours.

Reducing sugar determinations of the hydrolyzates were done according to the method described on page 23.

Sample	Mg. of Reducing Sugar per 0.06 g. Normal Meconium
Untreated	4.2 mg.
$Ba(OH)_2$-$ZnSO_4$	3.6 mg.
$Pb(OAc)_2$	0.8 mg.

Precipitation from the barium hydroxide-zinc sulfate supernatant with either alcohol or glacial acetic acid yielded fairly white precipitates in both cases, while similar precipitates from an untreated meconium suspension retained a great deal of color.

Because of the higher yield and fairly white precipitate that was obtained with the barium hydroxide-zinc sulfate treatment, that method seemed the best to use for removing initial protein and water-insoluble impurities.

C. Barium Hydroxide-Zinc Sulfate Purification of Normal Meconium.

1. Preparation. Ten grams of the mixed meconium from three normal babies were suspended in 100 cc. of water in a Waring blendor. 30 cc. of zinc sulfate and 30 cc. of barium hydroxide solutions were added, and after careful mixing, the precipitate was separated by centrifugation. The precipitate was washed once with 25 cc. of water. The combined

supernatants were then treated with 5 cc. of zinc sulfate and 5 cc. of barium hydroxide solutions and centrifuged.

The supernatants were evaporated under vacuum to about 90 cc. A small sample was removed for later analysis, and 95% alcohol was slowly added to the remainder. Two volumes caused significant precipitation. A total of four volumes of alcohol were added.

After standing overnight in the ice-box, it was centrifuged, and the precipitate washed once with 95% alcohol. The precipitate was then dissolved in 15 cc. of water and reprecipitated with 150 cc. of glacial acetic acid. This precipitate, although quite small in amount, had very little color. The supernatant had a decided green color.

The precipitate was washed once with 91% acetic acid, and then redissolved in 10 cc. of water. When this solution was treated with 110 cc. of glacial acetic acid, a more flocculent precipitate formed. This was washed once with 91% acetic acid, three times with 95% alcohol, once with ether, and once with a mixture of chloroform and ether. It was dried in vacuum over potassium hydroxide. Yield 0.61 g.

After the acetic acid solution was evaporated to dryness, redissolved in water, and again dried, there remained 0.75 g. of solid material. The alcohol supernatant, contained 0.45 g. of solid material.

Suspensions of each of the materials were prepared for analysis.

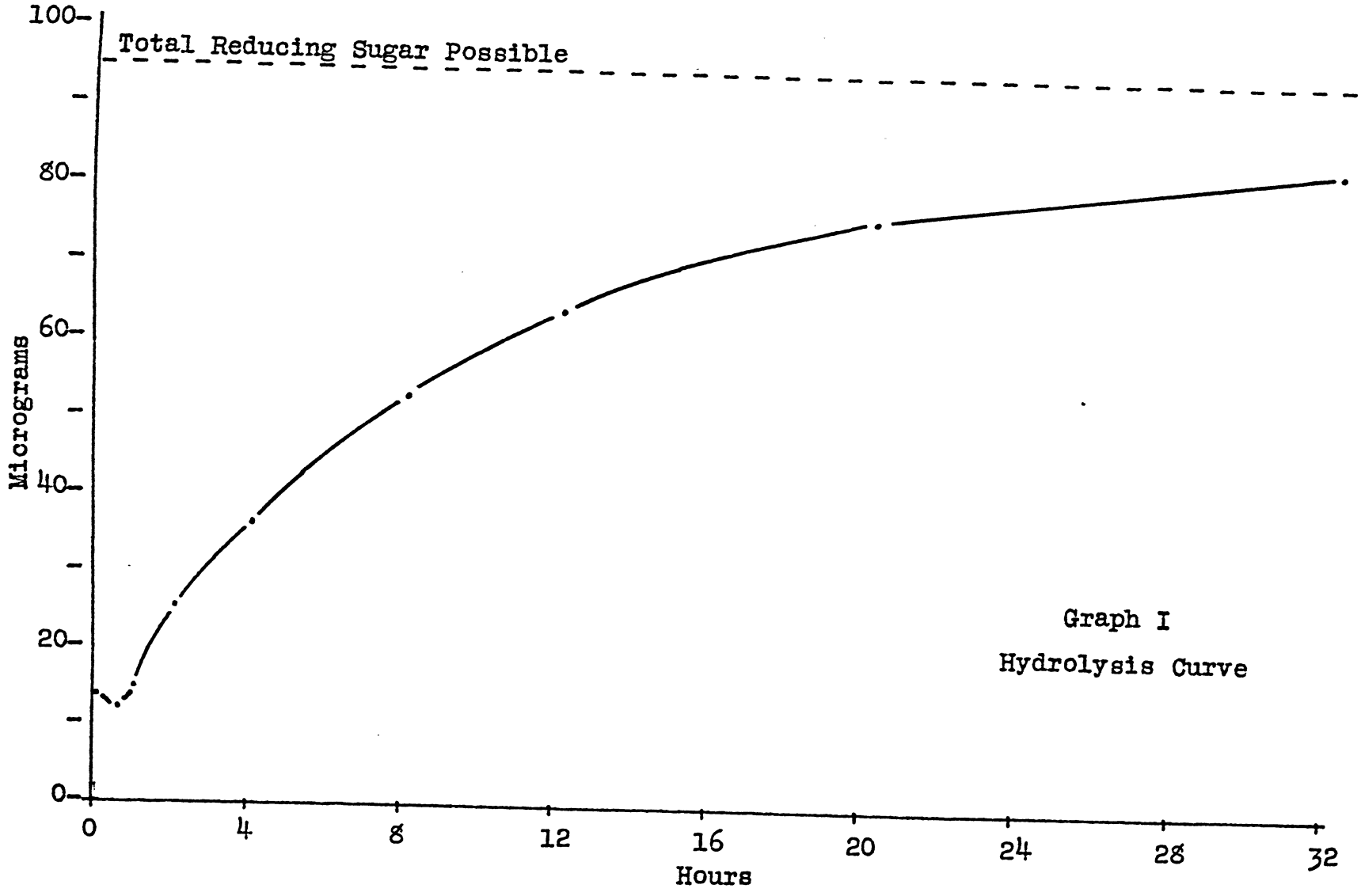
2. Determination of Reducing Sugar. Samples of the polysaccharide suspension, the alcohol and acetic acid residue suspensions, and the solution after deproteinization were analyzed for their reducing sugar content according to the method described on page 23.

Results:

Sample	Total R. S. g.	Total Weight g.	R. S. %	Yield %
Solution after deproteinization	0.400	----	----	----
Polysaccharide	0.196	0.61	32.1	49.0
Acetic acid residue	0.076	0.75	10.1	19.0
Alcohol residue	0.097	0.45	21.6	24.2
Reducing sugar recovered	0.369			92.2

A sample of the supernatant after the treatment of a meconium suspension with barium hydroxide and zinc sulfate was made 0.1 N with respect to hydrochloric acid and hydrolyzed on a boiling water bath, using an air condenser to prevent evaporation. Samples of the hydrolysis mixture were removed after 1/2, 1, 2, 4, 8, 12, 20, 32, and 70 hours. They were neutralized, and reducing sugar determinations were run on appropriate aliquots.

The hydrolysis curve with reducing sugar values plotted against hours of hydrolysis was a smooth curve reaching a



Graph I

Hydrolysis Curve

constant reducing sugar value after 32 hours, see graph I.

When the logarithm of the concentration of reducing sugar was plotted against the number of hours of hydrolysis, a virtually straight line was formed, which would indicate a pseudo-first order reaction. This would imply that the linkages were all of one type. See graph II.

3. Determination of Dry Weight. A sample of the polysaccharide was carefully weighed into a tared vessel, after the polysaccharide had been exposed to room atmosphere for two hours. It was then dried in a drying pistol heated with boiling water and evacuated with a vacuum pump for two hours.

Results: 5.2% of the material was moisture. The material was then allowed to stand at room atmosphere for several hours, during which time it absorbed more moisture than it had originally contained (there was more moisture in the atmosphere than when it had originally been allowed to equilibrate with the room atmosphere).

4. Determination of the Total Nitrogen Content. Appropriate samples of the polysaccharide suspension were digested and analyzed for nitrogen by the micro-Kjeldahl method described on page 43.

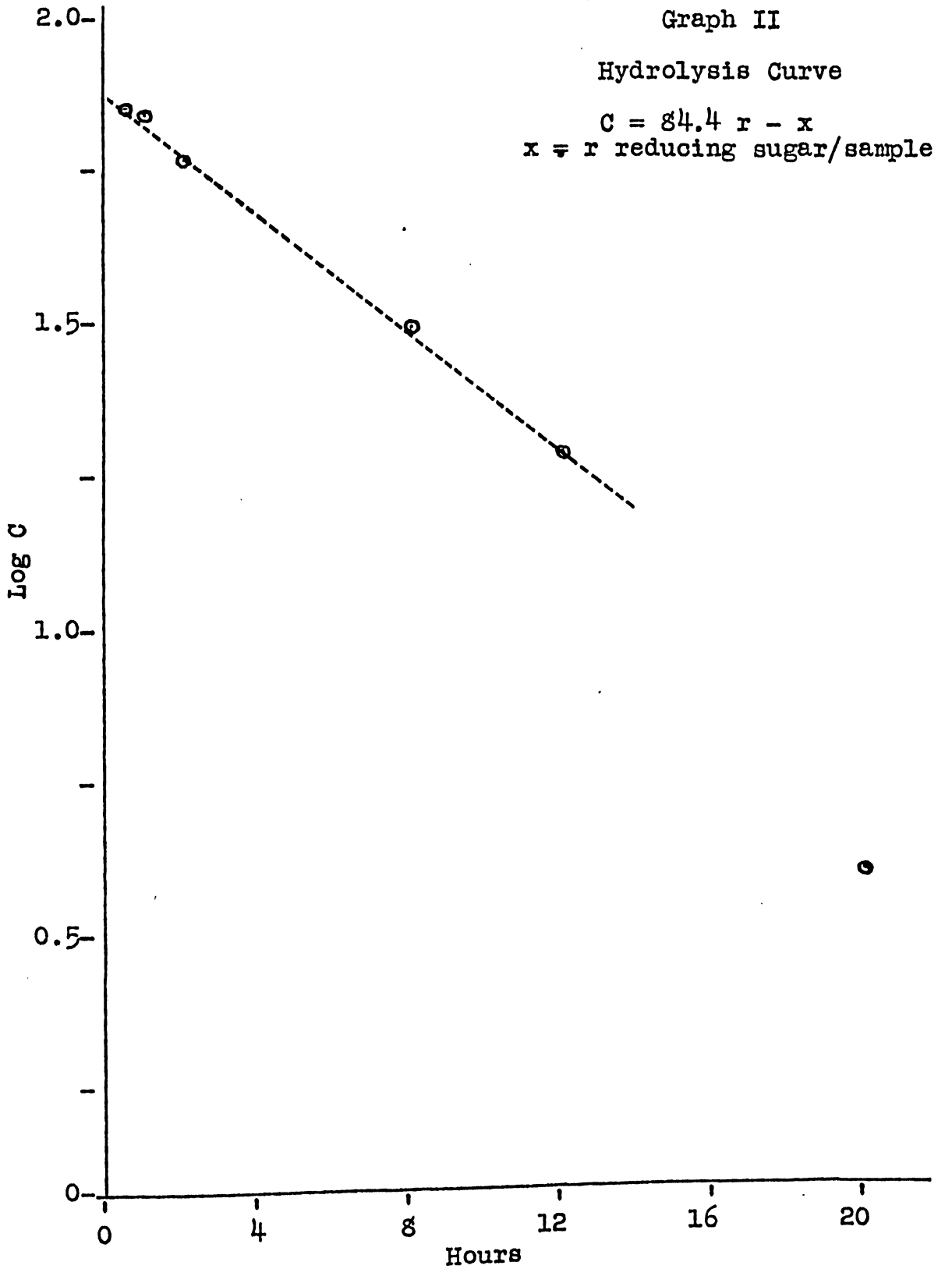
Results: 4.3% of the material was nitrogen.

Graph II

Hydrolysis Curve

$C = 84.4\ r - x$

$x = r$ reducing sugar/sample



5. Determination of the Amino Sugar Content. Appropriate samples of the polysaccharide suspension were analyzed for amino sugar content by the method described on page 26. Results: 58.7% of the reducing sugar in the polysaccharide, calculated as glucose, was amino sugar. 18.8% of the polysaccharide was amino sugar.

Discussion: Since the reducing value of amino sugar was somewhat less than that of glucose, these results would indicate that the reducing sugar content of the polysaccharide was probably greater than the reducing sugar values indicated and that even more of the polysaccharide was probably amino sugar. At least there was no doubt that amino sugars comprised the largest reducing sugar component of the polysaccharide.

6. Determination of Uronic Acid.

a. A qualitative test for uronic acids was performed by heating a sample of the polysaccharide solution with an equal volume of concentrated hydrochloric acid and adding a little α-naphthoresorcinol. The mixture was again heated and cooled. A layer of ether was placed above it, which became a bluish-rose, indicating the presence of uronic acid. A repeat test did not give this indication.

b. Although fairly accurate results could be obtained with a standard sample of galacturonic acid using the CO_2

liberation method, page 32, for the quantitative estimation of uronic acid, the values obtained with the polysaccharide were all very close to the blank values, and gave no indication of the presence of even traces of uronic acid.

Using the colorimetric method for the quantitative estimation of uronic acids, described on page 32, both the unhydrolyzed and hydrolyzed polysaccharide gave results which were identical with the blanks, indicating that there was no free uronic acid present in either case to react with the naphthoresorcinol reagent.

7. Determination of Sulfate Content. 28.2 mg. of the polysaccharide were treated with 2.0 cc. of 1 N hydrochloric acid and hydrolyzed on a boiling water bath for two hours in order to free any combined sulfate. The solution, which contained considerable amounts of insoluble material, was centrifuged and the precipitate washed five times with water. The supernatants were combined in a tared centrifuge tube and treated with a 25% barium chloride solution. The mixture was heated for an hour on a water bath, then centrifuged, washed four times with 50% alcohol, and dried in an oven at 110° C. The barium sulfate, which had a very dirty look, was weighed.

Results: 2.3% sulfate in the polysaccharide. This result was undoubtedly high, but even so was not considered significant. The sulfate found was probably due to traces of the

protein precipitating reagents.

8. Determination of Acetyl Content. The acetyl content was determined according to the method described on page 51.
Results: 6% acetyl in the polysaccharide.
Discussion: Frequently the acetyl groups are attached to the amino groups of amino sugars if they are present in the polysaccharide. This polysaccharide had 0.105 mol % of amino sugar and 0.140 mol % of acetyl groups or a ratio of 1.33 mol % acetyl groups to 1.0 mol % amino sugar content.

9. Determination of Pentose Sugars. A sample of the hydrolyzed polysaccharide was tested for the presence of pentose sugars according to the method described on page 30. The results were completely negative, giving no indication of the presence of pentoses.

10. Determination of the Hexose Content. The method described on page 37 was used for this determination.
Results:

Sugar Sample	Ratio Reading 520 mμ/Reading 420 mμ
Glucose Standard	1.90
Galactose Standard	1.08
Mannose Standard	0.57
Glucosamine Standard	(Sometimes values were slightly lower than blanks at 520 mμ, thus giving negative ratios.)
Polysaccharide	0.90

Calculating the 520 mμ value as being due to galactose alone, 20.7% of the polysaccharide was estimated to be

galactose.

Discussion: The ratios would seem to indicate that a good part of the hexose content of the polysaccharide was galactose. Since glucosamine appeared to depress the ratio, it would seem possible that the polysaccharide was composed largely of galactose and amino sugar.

This estimated value for galactose plus the value for amino sugars would more than account for the value of reducing sugar, calculated as glucose. If the reducing sugar value were calculated on the basis of galactose or amino sugar, it would more nearly equal the sum of galactose and amino sugar values.

Reducing sugar calculated as glucose	32.1%
Reducing sugar calculated as galactose	39.4%
Reducing sugar calculated as glucosamine	35.2%
Amino sugar + galactose = 18.8% + 20.7% =	39.5%

These figures would imply that the reducing sugar values and amino sugar and galactose values were fairly compatible and account for most of the reducing sugars present in the polysaccharide.

11. Determination of Methyl Pentose. The method described on page 28 was used for this determination. Results: With three minutes heating, 4.5% methyl pentose was found in the polysaccharide. With the ten minute heating, there was a definite indication of methyl pentose, but the

amount was not determined. Graph III shows the density of the products plotted against mμ after the three minute heating.

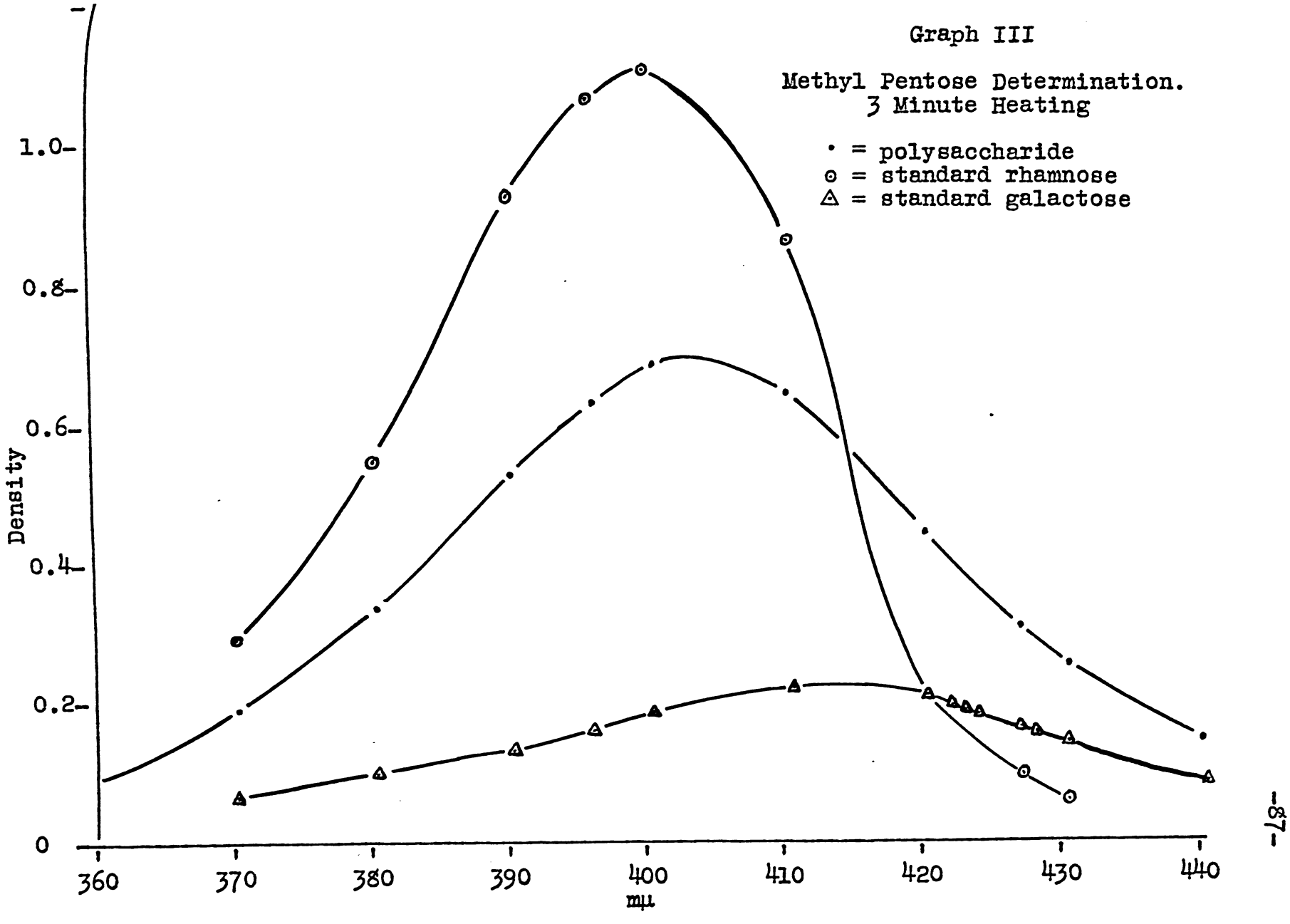
Discussion: This gave definite evidence for the presence of methyl pentose, but the exact amount may not be too accurate, because of the great number of possibilities for error that exist in the method.

12. Qualitative Protein Tests (85).

a. Xanthoproteic test. Two drops of the polysaccharide suspension were boiled with one drop of concentrated nitric acid. Upon cooling, it was made alkaline. There was a definite yellow color, which is a positive test indicating the presence of a phenyl group in the polysaccharide-amino acid complex. Probably this was due either to tyrosine or tryptophan.

b. Voisenet-Rhode Reaction. Two drops of the polysaccharide suspension were treated with two drops of Ehrlich's reagent, see page 27, mixed, and layered on top of concentrated sulfuric acid. No colored ring was visible. The presence of a red-violet color in this test was evidence for the presence of tryptophan.

c. Millon's Test. One gram of mercury was dissolved in 1.4 cc. of concentrated nitric acid and diluted with 3 cc. of water. One drop of this mixture was added to three drops of the polysaccharide suspension and heated. No color


Graph III
Methyl Pentose Determination.
3 Minute Heating
• = polysaccharide
⊙ = standard rhamnose
△ = standard galactose
Density
1.0
0.8
0.6
0.4
0.2
0
360
370
380
390
400
410
420
430
440
mμ

developed, thus indicating the absence of tyrosine in the material.

d. Biuret Test. One drop of 10 N sodium hydroxide, two drops of polysaccharide suspension, and two drops of a 0.1% copper sulfate solution were mixed together. A definite purplish color developed, indicating that the sample contained two carbamyl ($-CONH_2$) groups joined either directly together or through a single atom of nitrogen or carbon.

13. Conclusions.

Summary of Data on Polysaccharide Prepared from $Ba(OH)_2$-$ZnSO_4$ Supernatant

Component of the Polysaccharide		% of Total
Moisture		5.2
Reducing sugar (calculated as glucose)	32.1	
Amino sugar		18.8
Galactose		20.7
Methyl pentose		4.5
Nitrogen	4.3	
Amino sugar nitrogen	1.5	
Protein nitrogen	2.8	
Protein*		17.5
Sulfate		2.3
Acetyl		6.0
Total % recovered		75.0

* Protein nitrogen times the protein factor, 6.25 (see page 67), equals the amount of protein.

D. Sevag Purification of Normal Meconium.

1. Procedure. The Sevag procedure (86,32) employs certain organic liquids which deproteinize a solution when a thorough mixture is made. These substances also tend to remove those impurities which are soluble in organic solvents such as fat and various pigments.

a. First preparation.

A suspension of meconium in approximately ten times its weight of water was made in a Waring blendor. A small sample was removed for later analysis. Two grams of sodium acetate were added per gram of meconium and the pH adjusted to approximately 4.8 by adding 20% acetic acid. Acutint papers* were used to determine the pH externally.

A mixture of ten parts carbon tetrachloride to one part normal amyl alcohol were added in amounts equivalent to one-fifth the volume of the meconium mixture. This was shaken vigorously for one-half hour. After centrifuging, the supernatant was again shaken with another portion of the carbon tetrachloride-amyl alcohol mixture. This procedure was repeated at least three more times depending upon the amount of material removed by the Sevag reagents and upon the appearance of the supernatant.

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* Supplied by Anachemia Ltd., Montreal, Canada.

The final supernatant was then carefully treated with absolute alcohol. One-half volume of alcohol appeared to remove any turbidity that remained in the solution but further additions caused increasing cloudiness. A total of six volumes of absolute alcohol were added, causing complete precipitation. The precipitate had only a faint green color, but when dissolved in water it formed a somewhat darker solution which was translucent and quite viscous.

Yield of purified substance as based upon the recovery of reducing sugar (as determined by reducing sugar method, see page 23):

Reducing sugar in original suspension = 0.1525 g.
Reducing sugar in purified solution = 0.1212 g.
or approximately a 79.5% recovery of reducing sugar.

An attempt was made to further purify the material. A portion of the polysaccharide solution after Sevag purification was treated with ten volumes of glacial acetic acid. This caused turbidity but no appreciable precipitation, so the mixture was treated with an equal volume of absolute alcohol. A light, slightly green precipitate separated, leaving a clear supernatant. The precipitate was washed twice with absolute alcohol and then dissolved in water. This solution was a much lighter green but more opalescent than before.

Yield of purified substance as based on the recovery of reducing sugar:

Reducing sugar after Sevag purification = 42.5 mg.
Reducing sugar after HAc-alcohol purification = 36.7 mg.
or approximately an 86.3% recovery of reducing sugar.

Based upon the amino sugar content, the general character of the polysaccharide was not changed. The determination was carried out according to the procedure described on page 26.

Amino sugar before Sevag purification = 55%
Amino sugar after Sevag purification = 56%
Amino sugar after HAc-alcohol purification = 58% of the reducing sugar content.

b. Second Preparation.

This procedure was later repeated on a larger sample of meconium, which was from a one part meconium to two parts water suspension of the pooled meconium of twenty-five apparently normal babies.

The same general procedure was employed, the suspension being treated with the Sevag reagents seven times. The first three Sevag residues were combined and evaporated to dryness under vacuum, as were the last four. Each residue was suspended in water and treated with six volumes of absolute alcohol. The precipitates were then washed once with alcohol and dried in vacuum over potassium hydroxide.

The supernatant from the Sevag treatment was treated with six volumes of absolute alcohol. The precipitate was collected and washed four times with absolute alcohol and

dried under vacuum over potassium hydroxide.

Sevag I, the material from the first three Sevag treatments, was very dark in color and contained large amounts of lanugo hair and other impurities.

Sevag II, the material from the last four Sevag treatments, had a light green color and was fairly homogeneous.

Sevag III, the purified precipitate from the Sevag supernatant, had very little color as a solid.

Yield:

Original dry weight of meconium*	1.49 g.
Sevag I	0.45 g. or 30.2%
Sevag II	0.10 g. or 6.7%
Sevag III	0.74 g. or 49.7%
Original meconium recovered	1.29 g. or 86.6%

* Based on dry weight of the meconium suspension after drying in drying pistol at 100° C. under vacuum for two hours.

2. Characterization of the Products. The methods of determination were as follows: reducing sugar method as described on page 23; amino sugar method as described on page 26; methyl pentose method as described on page 28, using the ten minute heating; acetyl determination as described on page 51; and the Nessler nitrogen method as described on page 46.

The chemical composition of meconium and its purified products resembled the reported composition of blood-group substances, so it was of interest to see if they did possess

blood group activity. The method, as described on page 61, was used. The protein values were calculated, not determined.

Results:

Sample	Reducing Sugar %*	Amino Sugar %*	Methyl Pentose %*	Acetyl %*	Calcd. as Protein** %*	Total %*
Original	26.2	15.3	3.8	5.0	36.9	68.1
Sevag I	11.1	6.6	---	---	36.3	47.4
Sevag II	23.9	17.8	---	---	38.8	62.7
Sevag III	42.6	25.9	5.8	9.6	25.0	77.2

Sample	Total N %*	Amino Sugar %*	Amino Sugar N %*	Ratio A.S.N/ Total N	N Calcd. as Protein N %*	Calcd. as Protein** %*
Original	7.1	15.3	1.2	0.17	5.9	36.9
Sevag I	6.3	6.6	0.5	0.08	5.8	36.3
Sevag II	7.6	17.8	1.4	0.18	6.2	38.8
Sevag III	6.0	25.9	2.0	0.34	4.0	25.0

Sample	Amino Sugar %*	Mol % Amino Sugar	Acetyl %*	Mol % Acetyl	Mol % acetyl/ Mol % amino sugar
Original	15.3	0.085	5.0	0.116	1.36
Sevag III	25.9	0.145	9.6	0.223	1.54

Sample	Reducing Sugar %*	Amino Sugar %*	Methyl Pentose %*	N %*	Ratio A.S./ R.S.	Ratio M.P./ R.S.	Ratio R.S./ N
Original	26.2	15.3	3.8	7.1	0.586	0.145	3.8
Sevag I	11.1	6.6	---	6.3	0.595	---	1.8
Sevag II	23.9	17.8	---	7.6	0.744	---	3.1
Sevag III	42.6	25.9	5.8	6.0	0.608	0.136	7.1

* on dry weight basis
** % protein nitrogen times 6.25, see page 67.

Sample	Blood Group Activity* A	B
First preparation		
Original meconium suspension	3.0	--
After Sevag purification	0.2	--
After HAc-alcohol purification	1.1	--
Second preparation		
Original meconium suspension	2.5	30
Sevag I	6.2	62
Sevag II	1.0	100
Sevag III	0.1	16

* The approximage amount of material inhibiting isoagglutination, expressed in μg. in 0.1 cc. of solution tested. The dashes indicate lack of activity with the highest amount tested, usually 1,000 μg.

Discussion: In the data presented above there were several points of special interest.

a. The percentage of reducing sugar, amino sugar, methyl pentose, and acetyl in the products rose with the amount of purification. This would indicate that the carbohydrate in meconium was largely an essential part of the purest fraction, and not present as an impurity. This was verified, as shown below:

Sample	Weight of Sample g.	Weight of Reducing Sugar g.	Weight of Amino Sugar g.
Original	1.49	0.390	0.228
Sevag I	0.45	0.050	0.030
Sevag II	0.10	0.024	0.018
Sevag III	0.74	0.315	0.192
% of Original Recovered	86.6%	100%	105%

b. The nitrogen content of the purest fraction was less than the original suspension and other less pure fractions, which indicated that at least part of the impurities removed during the purification process contained nitrogen and were likely proteins or breakdown products of proteins.

Sample	Weight of Sample g.	Weight of Nitrogen g.
Original Meconium Suspension	1.49	0.106
Sevag I	0.45	0.028
Sevag II	0.10	0.008
Sevag III	0.74	0.044
Original Recovered	86.6%	75.4%

c. The reason for the loss of nitrogen was possibly due to the fact that it was present as an impurity, separate from the carbohydrate component and soluble in the water-alcohol supernatants. The fact that the total nitrogen content was larger and duplicate samples were always run would indicate that the method of determination was satisfactory.

d. One reason that the percentage of the original meconium accounted for was low was that the 10% fat content was not taken into account.

e. The ratio of amino sugar nitrogen to the total nitrogen content was lower in Sevag I than the original meconium suspension. The ratio for Sevag II was about the same as the original, while for Sevag III, it was consider-

ably larger. The values for amino sugar nitrogen percentage varied in the same way.

f. Sevag purification greatly increased the blood group activity per unit weight. Although the impure fractions were far less active, they still contained considerable activity. The acetic acid-alcohol treatment appeared to destroy some of the activity.

E. Phenol Purification of Normal Meconium.

1. Procedure. The phenol procedure was based on the method described by Morgan (55), in which he fractionated blood group substance by means of 90% phenol solution. There was a phenol insoluble portion, and a series of fractions obtained by the careful addition of absolute alcohol.

The method was done on a portion of the original mixed meconium suspension, described on page 91.

a. First preparation. 15 g. of the meconium suspension were treated with 90 g. of solid phenol and 100 cc. of a 90% phenol solution. This was thoroughly mixed and then heavily centrifuged for two hours, yielding a little insoluble material which had a gel-like consistency and contained some lanugo hairs. This was washed four times with absolute alcohol and dried under vacuum over potassium hydroxide. This changed its appearance to a dry mass of lanugo hair mixed with a powdery material.

The supernatant was treated with enough 1:1 absolute alcohol:phenol mixture and absolute alcohol to bring the alcohol concentration to approximately 25% alcohol. After standing overnight, the mixture was centrifuged. The resulting precipitate was resuspended in 90% phenol, and sufficient 1:1 alcohol:phenol mixture added to make the alcohol concentration approximately 15%. The precipitate was separated, washed four times with absolute alcohol, and dried in vacuum over potassium hydroxide.

The supernatant of the 25% alcohol precipitation was then treated with enough alcohol to bring the alcohol concentration to 50%. The addition of a little sodium acetate and acetic acid was necessary to stimulate flocculation of the precipitate. The precipitate was separated by centrifugation and redissolved in 90% phenol.

The supernatant of the 15% alcohol precipitation of the first fraction was added to this, and the alcohol concentration was adjusted to approximately 50%. The precipitate was separated by centrifugation and washed four times with absolute alcohol.

Yield:

Original dry weight of meconium*	1.47 g.
Precipitate I, insoluble in 90% phenol	0.56 g. or 38.1%
Precipitate II, pptd. by 15% alcohol	0.11 g. or 7.5%
Precipitate III, pptd. by 50% alcohol	0.60 g. or 40.8%
Original meconium recovered	1.27 g. or 86.4%

* Based on dry weight of the meconium suspension after drying in drying pistol at 100° C. under vacuum for two hours.

b. Second Preparation. The same general procedure was followed as described above, using 45 g. of the mixed meconium suspension.

The phenol-insoluble portion was washed with repeated portions of water yielding some phenol- and water-insoluble material, and a very viscous water solution. This solution yielded a definite precipitate when it was made 90% with respect to phenol. The supernatant from this did not give a precipitate when it was made 50% with respect to alcohol.

The main supernatant from the phenol-insoluble material was treated with absolute alcohol. Precipitates were collected at 15, 27, 40, and 67% alcohol concentrations. The further addition of alcohol did not cause any more precipitation. The various precipitates were washed with alcohol and dried in vacuum over sodium hydroxide.

The precipitate from the 90% phenol insoluble-water soluble fraction was redissolved in water and dialyzed against distilled water for twenty-four hours. The solution was very viscous and not completely homogeneous.

Yield:

Original dry weight of meconium*	4.37 g.	
Precipitate I, insoluble in 90% phenol and water	0.13 g.	or 3.0%
Precipitate II, insoluble in 90% phenol, soluble in water**	1.02 g.	or 23.3%
Precipitate III, pptd. by 15% alcohol	0.81 g.	or 18.5%
Precipitate IV, pptd. by 27% alcohol	0.62 g.	or 14.2%
Precipitate V, pptd. by 40% alcohol	0.29 g.	or 6.6%
Precipitate VI, pptd. by 67% alcohol	0.13 g.	or 3.0%
Original meconium recovered	3.00 g.	or 68.6%

* Based on dry weight of the meconium suspension after drying in drying pistol at 100° C. under vacuum for two hours.

** Weight approximated from reducing sugar value after dialysis, assuming 35% reducing sugar. Therefore there is probably considerable error in this figure.

2. Characterization of the Products. The methods of determination were as follows: reducing sugar method as described on page 23; amino sugar method as described on page 26; methyl pentose method as described on page 28; except that the ten minute heating was used; acetyl method as described on page 51; nitrogen method as described on page 46; and the determination of blood group activity as described on page 61.

Results:
First Preparation

Sample	Reducing Sugar %*	Amino Sugar %*	Methyl Pentose %*	Acetyl %*	Calcd. as Protein** %*	Total %*
Original	26.2	15.3	3.8	5.0	36.9	68.1
Ppt. I	34.7	23.2	---	---	22.5	57.2
Ppt. II	43.9	28.4	6.0	8.9	18.1	70.9
Ppt. III	22.4	13.5	---	---	29.4	51.8

Sample	Total N %*	Amino Sugar %*	Amino Sugar N %*	Ratio A.S.N/ Total N	N Calcd. as Protein N %*	Protein** %*
Original	7.1	15.3	1.2	0.17	5.9	36.9
Ppt. I	5.4	23.2	1.8	0.33	3.6	22.5
Ppt. II	5.1	28.4	2.2	0.43	2.9	18.1
Ppt. III	5.8	13.5	1.1	0.19	4.7	29.4

Sample	Amino Sugar %*	Mol % Amino Sugar	Acetyl %*	Mol % Acetyl	Mol % acetyl/ Mol % Amino Sugar
Original	15.3	0.085	5.0	0.116	1.36
Ppt. II	28.4	0.159	8.9	0.207	1.30

Sample	Reducing Sugar %*	Amino Sugar %*	Methyl Pentose %*	N %*	Ratio A.S./ R.S.	Ratio M.P./ R.S.	Ratio R.S./ N
Original	26.2	15.3	3.8	7.1	0.586	0.145	3.8
Ppt. I	34.7	23.2	---	5.4	0.669	---	6.4
Ppt. II	43.9	28.4	6.0	5.1	0.647	0.137	8.6
Ppt. III	22.4	13.5	---	5.8	0.603	---	3.9

* On dry weight basis
** % protein nitrogen times 6.25, page 67. No actual protein determinations were made on these samples.

Sample	Blood Group Activity A	B
Original meconium suspension	2.5	30
Precipitate I	10 (5.8)**	25(14.6)**
Precipitate II	0.5	25
Precipitate III	2.5(1.5)**	250(150)**

* The approximate amount of material inhibiting isoagglutination, expressed in μg. in 0.1 cc. of solution tested.
** Precipitate I was only 58.4% soluble in water and precipitate II only 59.5% soluble. The figures in parenthesis represent the activity in the soluble fraction.

Second Preparation

The fractions of the second preparation which were precipitated by alcohol did not completely dissolve in water, so an attempt was made to dissolve them in normal saline and sodium carbonate. There was still some material which was insoluble, so the mixtures were centrifuged and the amount of soluble reducing sugar, nitrogen, and blood group activity determined.

Sample	Sol. Reducing Sugar mg.	Reducing Sugar Total mg.	Reducing Sugar Total %	Sol. Reducing Sugar %	Sol. N mg.	Ratio R.S./N
Ppt. II	---	28.4	---	---	4.13(total)	6.9
Ppt. III	15.1	15.6	36.1	96.8	1.91	7.9
Ppt. IV	3.9	6.4	17.4	60.9	0.85	4.6
Ppt. V	5.95	7.1	20.5	83.8	1.14	5.2
Ppt. VI	8.8	9.9	22.5	88.8	2.10	4.2

Sample	Blood Group Activity* A	B
Original Meconium Suspension	2.5	30
Precipitate II	120	12
Precipitate III	8.4	8.4
Precipitate IV	45	>45
Precipitate V	58	>58
Precipitate VI	>780	>780

* The approximate amount of material inhibiting isoagglutination, expressed in µg. in 0.1 cc. of solution tested.

Discussion: In the results there were several very interesting points:

a. In both preparations the precipitate which had the highest percentage of carbohydrate was that which precipitated with approximately 15% alcohol. In the first preparation, this fraction also had the highest amino sugar content.

b. The 15% alcohol precipitate likewise had the highest reducing sugar to nitrogen ratio. However, the 15% alcohol precipitate did not amount to more than a 15% recovery from the original meconium.

c. This same fraction in the first preparation also had the lowest per cent of nitrogen.

Sample	Total Weight g.	Reducing Sugar %	Weight Reducing Sugar g.	Amino Sugar %	Weight Amino Sugar g.	N %	Weight N g.
Original	1.47	26.2	0.39	15.3	0.23	7.1	0.104
Ppt. I	0.56	34.7	0.19	23.2	0.13	5.4	0.030
Ppt. II	0.11	43.9	0.05	28.4	0.03	5.1	0.006
Ppt. III	0.60	22.4	0.13	13.5	0.08	5.8	0.035
% original recovered	86.4%		94.9%		104.3%		68.3%

d. One explanation for the low per cent of the original meconium accounted for is the fact that no allowance was made for the 10% fat content.

e. As with the Sevag preparations these results would seem to indicate that the carbohydrate portion was present as an essential portion of the purified substance and was not present as an impurity.

f. The reason for the loss of nitrogen was probably due to the fact that it was present as an impurity, separate from the carbohydrate component, and possibly soluble in the alcohol-phenol supernatants. The fact that the total nitrogen content was larger and duplicate samples were always run would indicate that the method of determination was satisfactory.

g. This method of preparation seemed to be very drastic as evidenced by the large amount of material that became insoluble in water. This could be due either to a type of denaturation or to an extreme dehydration of the products. However, Kabat and his co-workers found that if the 90% phenol mixture were perfectly clear and had no lipid layer on top, there was less insoluble material. Possibly this was part of the cause of so much insoluble material.

h. Another evidence for the harshness of the procedure was shown by the relatively low amount of blood group activity that could be demonstrated in the products. This was

particularly evident in the second preparation.

i. In both preparations there was an indication that there might possibly be some separation of A and B activity by this method. However it was still far from complete.

j. Morgan and King (55) had used this method on hog gastric mucin originally and obtained the following results:

Sample	% of Original	Nitrogen %	Blood Group Activity*
Insoluble in 90% phenol	25.4	6.9	10-100
Precipitated with 0-10% alcohol	26.3	5.5	0.1
Precipitated with 10-25% alcohol	14.3	8.0	1.0
Precipitated with 25-50% alcohol	9.3	12.2	10
Supernatant	10.1	10.4	---
Recovery	85.4		

* Approximately the minimum amount of material in µg. completely inhibiting isoagglutination of A cells.

The blood group activity indicated that the fractions of mucin and meconium were similar with the largest amount of activity being present in the fraction of material precipitating between 0-15% alcohol.

F. Ammonium Sulfate Fractionation of Normal Meconium.

1. First Preparation

a. Procedure. A sample of the original meconium suspension, as described on page 91, was centrifuged to remove any water-insoluble material. The supernatant was then treated

in the cold room, about 38° F., with an equal volume of saturated ammonium sulfate solution. This caused no precipitation, so the ammonium sulfate concentration was gradually increased to 75% saturation. At this point solid ammonium sulfate was added to bring the entire mixture to complete saturation. This caused a very definite precipitation. After standing overnight, the precipitate was separated by filtering with suction. The filtrate was quite clear and had a light yellow color.

The precipitate was dissolved in water. Both the dissolved precipitate and the supernatant were dialyzed against distilled water for eighteen hours.

b. Characterization of the Products. These solutions, after dialysis, were analyzed for reducing sugar and nitrogen content according to the methods described on page 23 and page 46.

Results:

Sample	N mg.	N %	Reducing Sugar mg.	Reducing Sugar %	Ratio R.S./N
Theoretical Starting Amount	16.6	---	61.1	---	3.68
I. Precipitate	5.7	34.3	20.8	34.0	3.65
II. Supernatant	5.1	30.7	9.4	15.4	1.84
Recovery	10.8	65.0	30.2	49.4	---

Discussion: Normal meconium was not precipitated by ammonium sulfate until after a seventy-five per cent concentration was

reached. This was in decided contrast to meconium ileus which was almost completely precipitated with that concentration of ammonium sulfate.

The supernatant of the saturated ammonium sulfate precipitate contained large amounts of carbohydrate and nitrogen in contrast to that of meconium ileus which contained only a little nitrogen and no carbohydrate.

The reducing sugar to nitrogen ratio of the precipitate was approximately the same as that of the original meconium suspension.

The nitrogen values were possibly in error because the dialysis was not complete and there were large amounts of inorganic nitrogen still present. Also there was some indication that the reducing sugar determination in the supernatant was somewhat inhibited by the presence of too much neutral salt.

2. Second Preparation.

a. Procedure. A sample of the original meconium suspension was diluted with water and centrifuged to remove any water insoluble material. 4.5 cc. of the supernatant were then treated with 0.25 cc. of water and 0.25 cc. of 2 M acetate buffer, pH 5.65.

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* 0.5 cc. of 2 N acetic acid was added to 5 cc. of 2 M sodium acetate.

Saturated ammonium sulfate solution was gradually added to the meconium mixture in the cold room, about 38° F. No significant precipitation occurred until the concentration was brought to 83%. The precipitate was separated by centrifugation.

When more saturated ammonium sulfate solution was added, no further precipitation occurred, so solid ammonium sulfate was added to saturate the mixture. This caused some precipitate to form. It was separated by heavy centrifugation.

The precipitates were dissolved in water. These solutions and the supernatant were all dialyzed against distilled water for twenty-six hours.

b. Characterization of the Products.

Results:

Sample	Reducing Sugar mg.	% of Total
Theoretical Starting Amount	22.9	----
I. 83% precipitate	3.8	16.6
II. Saturated, precipitate	4.6	20.1
III. Supernatant	2.6	11.3
Recovery	11.0	48.0

The non-dialyzable portion of fraction I, the 83% ammonium sulfate precipitate was characterized as follows:

1) One sample was treated with 2 M acetate buffer, pH 5.65, and then with a saturated solution of ammonium sulfate in the cold room. No precipitation occurred until a

saturation of 83% was reached. This gave a definite precipitate and a water clear supernatant. The precipitate was analyzed for reducing sugar content.

2) Another sample was treated with 2 M acetate buffer, pH 5.65, and then with absolute alcohol in the cold room. No precipitation occurred until the solution had a 60% alcohol concentration. The precipitate was separated by centrifugation, and the supernatant treated with more alcohol.

Small definite precipitates separated with alcohol concentrations of 67, 75, and 83%. Each precipitate was analyzed for reducing sugar content.

The non-dialyzable portion of fraction II, the saturated ammonium sulfate precipitate, was characterized as follows:

1) One sample was treated with 2 M acetate buffer, pH 5.65, and then with a saturated solution of ammonium sulfate in the cold room. No precipitation occurred with a saturation of 83%, so the solution was saturated with solid ammonium sulfate. This gave a definite precipitate and a water-clear supernatant. The precipitate was analyzed for reducing sugar content.

2) Another sample was treated with 2 M acetate buffer, pH 5.65, and then with absolute alcohol in the cold room. Only a slight cloud formed but no definite precipitate until the alcohol concentration reached 67%. Definite precipitates were obtained with 67 and 75% concentrations. The further

addition of alcohol caused no further precipitation. The two precipitates and the supernatant were analyzed for reducing sugar content.

Results:

Sample	Reducing Sugar µg.	% of Total
I. 83% Ammonium Sulfate Fraction		
A. Alcohol Fractionation		
Theoretical Starting Amount	1400	----
I. 60% Alcohol	339	24.2
II. 67% Alcohol	171	12.2
III. 75% Alcohol	390	27.9
IV. 83% Alcohol	500	35.7
V. Supernatant	40	2.9
Recovery	1440	102.9
B. Ammonium Sulfate Fractionation		
Theoretical Starting Amount	1400	----
I. 83% Ammonium Sulfate	555	39.6
II. Saturated Ammonium Sulfate Fraction		
A. Alcohol Fractionation		
Theoretical Starting Amount	1870	----
I. 67% Alcohol	336	18.0
II. 75% Alcohol	1210	64.7
III. Supernatant	64	3.4
Recovery	1610	86.1
B. Ammonium Sulfate Fractionation		
Theoretical Starting Amount	1870	----
I. Saturated Ammonium Sulfate	435	23.0

Discussion: From the reducing sugar values it appeared that the ammonium sulfate precipitation was fairly reproducible, with the largest amount precipitating with saturated ammonium sulfate and the rest remaining in the supernatant. With slower addition of ammonium sulfate, it was shown that about a half of the precipitate came down at a saturation of 83%.

When repeated precipitations of the 83% and saturated ammonium sulfate fractions were made, only very incomplete precipitation occurred.

Alcohol fractionation of these samples also gave a series of precipitates with alcohol concentrations from 60 to 83%.

It was indicated therefore that the ammonium sulfate fractionation was not as clean or as reproducible as the alcohol fractionation of normal meconium.

Morgan and King (55) fractionated a sample of a material obtained from hog gastric mucin with ammonium sulfate. It originally had 5.7% N and $[\alpha]_{5461}$ $+12^{\circ}$. Up to 72% only a small amount of material separated which was similar to the original preparation. 55% of the material separated at 81% saturation. It was collected, dialyzed, precipitated with alcohol, and dried in vacuum. The $[\alpha]_{5461}$ of $+13^{\circ}$ and nitrogen content of 5.4% were similar to the original. The supernatant, after dialysis and recovery of the material yielded 12% of the total starting amount, which had low serological activity, $[\alpha]_{5461}$ -14° and a nitrogen content

of 4.3%

Our results compared very favorably with these.

G. Dialysis of Normal Meconium.

1. Preparation. One cc. of the original meconium suspension, as described on page 91, was diluted with 1.5 cc. of water and dialyzed against 20 cc. of distilled water for twenty-seven hours.

A sample of the non-dialyzable portion was again dialyzed against distilled water for sixteen hours in order to determine if the dialyzable portion of normal meconium was present in a definite concentration or if the dialysis was a gradual transfer which came to equilibrium.

2. Characterization of the Products. Appropriate aliquots were analyzed for reducing sugar and nitrogen contents, according to the methods described on page 23 and page 46. A sample of the dialyzable portion was tested according to the methods described on page 61, to see if it possessed any blood group activity.

Results:

Sample	Reducing Sugar mg.	Reducing Sugar %	N mg.	N %	Ratio R.S./N
Theoretical Starting Amount	26.6	----	7.1	----	3.75
I. Non-dialyzable	20.8	78.2	4.37	61.5	4.76
II. Dialyzable	3.5	13.2	0.76	10.7	4.61
Recovery	24.3	91.4	5.13	72.2	----

Sample	Reducing Sugar	Reducing Sugar
Theoretical Starting Amount	10.4	----
I. Non-dialyzable, after repeat dialysis	9.7	93.2
II. Dialyzable	0.3	2.9
Recovery	10.0	96.1

3. Characterization of the Dialyzable Portion. The dialyzable portions of normal meconium were evaporated in vacuum to a volume of 2.5 cc.

One aliquot was analyzed directly for free reducing sugar content and total reducing sugar content.

A 0.5 cc. sample was treated with 4.5 cc. of phenol, thus making the phenol concentration 90%. This gave a slight precipitate which was separated. The supernatant was treated with an equal volume of absolute alcohol which did not seem to cause any change. The 90% phenol precipitate was analyzed for total reducing sugar content.

Another aliquot was treated with four volumes of absolute alcohol, yielding only a minimal amount of precipitate which was analyzed for total reducing sugar content. The supernatant was treated with several drops of a saturated lead subacetate solution. This gave a definite precipitate which was separated by centrifugation. It was suspended in water and treated with a stream of hydrogen sulfide. The

precipitate was removed by centrifugation, resuspended in water, and treated with a stream of hydrogen sulfide. The precipitate was again removed by centrifugation. The combined hydrogen sulfide supernatants were analyzed for total reducing sugar content.

Results:

Sample	Reducing Sugar µg.	% of Total
Concentrated Dialyzable	520	----
Free Reducing Sugar	98.3	18.9
Phenol Insoluble	16.9	3.3
Alcohol Precipitate	24.8	4.8
H_2S Supernatant	200.8	38.6

Discussion: About 13% of the reducing sugar content of normal meconium was dialyzable. Morgan (55) reported that a specimen of crude gastric mucin was approximately 20-23% diffusible through a cellophane membrane.

When dialysis of the non-dialyzable portion was repeated only a minimal amount of reducing sugar diffused. This indicated that the non-dialyzable portion was a definite fraction which did not diffuse gradually or come to equilibrium with the dialyzing medium.

The reducing sugar to nitrogen ratio was higher after dialysis than before, possibly indicating the removal of amino acids and small protein fragments which were not essential components of the material composing the greater part of meconium.

Attempts were then made to characterize the dialyzable portion of normal meconium. 19% of the reducing sugar content was found to be free. Possibly this was either free reducing monosaccharides or small polysaccharides with many free reducing groups and of a small enough size to be easily dialyzed.

The greater part of the reducing sugar content of the dialyzable portion was phenol-alcohol and alcohol soluble. Most of it could be precipitated by lead subacetate. Probably much more was precipitated than could be demonstrated, because other work showed that the lead sulfide probably absorbed reducing sugars to a considerable extent.

Another indication that the dialyzable portion consisted mainly of reducing sugar and nitrogen fragments from the breakdown of proteins and polysaccharides was that it possessed no blood group activity, while normal meconium showed very high blood group activity. This activity is known to be due to a specific polysaccharide-amino acid complex.

H. Alcohol Fractionation of Normal Meconium.

1. First Fractionation.

a. Procedure. A sample of the original meconium suspension, as described on page 91, was diluted with water and centrifuged to remove the water insoluble material. 95% alcohol was gradually added to the suspension in the cold room, 38° F. The first precipitate separated at an alcohol

concentration of 56%. This was dark and not too readily soluble in water. Some of this could be separated by centrifugation as a mucus-type clot, but the supernatant could not be decanted.

At 62% alcohol concentration another fairly large amount of precipitate separated. This also formed a mucus-like clot and could not be drained. It was only slowly miscible with water.

With 66.5% alcohol, another small precipitate formed, which was readily soluble in water.

The supernatant was made 81% with respect to alcohol and a few crystals of sodium acetate were added to hasten flocculation. This was centrifuged, and the precipitate redissolved in water.

b. Characterization of the Products. Each precipitate and the supernatant were analyzed for reducing sugar and nitrogen content according to the methods described on page 23 and page 46, and blood group activity as described on page 61.

Results:

Sample	Reducing Sugar mg.	% of Total	N mg.	% of Total	Ratio R.S./N	Blood Group Activity* A	B
Theoretical Starting Amount	104	----	28	----	3.71	0.7	7.9
I. 56% Alc.	2.4	2.3	1.2	4.3	2.00	7.0	7.0
II. 62% Alc.	26.7	25.7	6.2	22.2	4.31	---	---
III. 66.5% Alc.	1.5	1.4	0.5	1.8	3.00	---	---
IV. 81% Alc.	43.3	41.6	7.4	26.4	5.85	12.4	12.4
V. Supernatant	13.1	12.6	3.2	11.4	4.09	---	---
Recovery	87.0	83.6	18.5	66.1	----	---	---

* The approximate amount of reducing sugar expressed in µg. present in the 0.1 cc. of solution tested, inhibiting agglutination.

Fraction II, the 62% alcohol fraction, contained some material which was not water soluble, so it was separated in a high speed centrifuge and analyzed. It contained 3.1 mg. of reducing sugar or 11.6% of the reducing sugar content of that fraction and 1.0 mg. of nitrogen or 16.1% of the nitrogen content of that fraction.

Fraction IV, the 81% alcohol fraction, was characterized as follows:

1) One sample of fraction IV was treated with phenol until it had a concentration of 90% phenol. The small amount of insoluble material was separated by centrifugation and drained. Alcohol was added to the supernatant until it was 15% alcohol. A precipitate was separated here and also with a 27% alcohol concentration.

The 90% phenol precipitate did not readily dissolve in water. The precipitate with 15% alcohol dissolved, but the solution was cloudy. The precipitate with 27% alcohol formed a clear solution. Each was analyzed for reducing sugar content.

2) Another sample of fraction IV was treated in the cold room with 95% alcohol. Precipitates were separated at alcohol concentrations of 62 and 81%. The addition of more alcohol and sodium acetate to the supernatant caused a large amount of precipitation, thus indicating that there may have been a pH or neutral salt affect which inhibited complete precipitation before.

Results:

Sample	Reducing Sugar mg.	% of Total
Theoretical Starting Amount	6.2	----
1) Phenol Fractionation		
I. Phenol Insoluble	3.0	48.4
II. 15% Alcohol	1.4	22.6
III. 27% Alcohol	0.8	12.9
Recovery	5.2	83.9
2) Alcohol Fractionation		
I. 62% Alcohol	1.3	21
II. 81% Alcohol	1.3	21
Recovery	2.6	42

The supernatant of the alcohol fractionation of normal meconium was characterized as follows:

1) Various portions were tested with the following reagents:

a) Glacial acetic acid caused no change.

b) Lead acetate caused no change.

c) Barium chloride caused no change.

d) Acetone caused a faint cloudiness in the solution.

e) Acetone plus ether made a distinct cloudiness.

f) Ether alone caused a heavy turbidity.

g) Lead subacetate caused the formation of a yellowish gelatinous precipitate.

2) A 30 cc. portion of the supernatant was treated with 1 cc. of a saturated lead subacetate solution. After checking for complete precipitation, the mixture was centrifuged and the precipitate drained. The precipitate was then resuspended in water and treated with a stream of hydrogen sulfide for ten minutes. The precipitate was separated by centrifugation, resuspended in water and treated with a stream of hydrogen sulfide. The precipitate was again separated by centrifugation.

The supernatants were combined. They were clear and seemed slightly viscous.

Portions of the solution were treated as follows:

a) Six volumes of 95% alcohol added to one volume of the supernatant caused no change. When ether was added to this, it caused a little turbidity, and more formed when

acetone was added.

b) Acetone and ether without alcohol also caused turbidity.

c) One portion was analyzed for reducing sugar and nitrogen content.

Results:

Sample	Reducing Sugar mg.	Nitrogen mg.	Ratio R.S./N
Theoretical Starting Amount	5.6	1.37	4.09
H_2S Supernatant	3.2	0.43	7.44
Recovery	57.1%	31.4%	----

Either the rest of the reducing sugar was not precipitated by the lead subacetate, was lost by handling, or was adsorbed by the lead sulfide. This also indicated that all of the carbohydrate and nitrogen constituents were not firmly bound together.

d) Another portion was aerated to remove any hydrogen sulfide remaining and tested for blood group activity. It gave no indication of either A or B activity.

e) A 0.5 cc. sample was dialyzed against 10 cc. of distilled water for sixteen hours at room temperature. Both the non-dialyzable and the dialyzable portion were analyzed for reducing sugar content.

Results:

Sample	Reducing Sugar µg.	% of Total
Theoretical Starting Amount	800	----
Dialyzable	374	46.8
Non-dialyzable	52	6.5
Recovery	426	53.3

Discussion: The alcohol fractionation of normal meconium gave two main fractions of precipitate, one at 62% and the other at 81% alcohol. When the 81% fraction was checked for homogeneity it was found to give several fractions with phenol and alcohol, and the repeat of the alcohol fractionation was very inconsistent. There was strong indication that the cause of this was a pH or neutral salt effect.

The supernatant of the alcohol fractionation did not have the same properties as the other fractions. A large amount of precipitate was produced by the addition of lead subacetate. Upon removal of the lead with hydrogen sulfide, the material which had been precipitated by lead subacetate was shown to contain both reducing sugar and nitrogen. Probably this would indicate that part of the reducing sugar and nitrogen were in some way combined and therefore precipitated together. This gave no indication of blood group activity and was almost completely dialyzable, thus showing that it consisted largely of fairly small molecules.

2. Second Fractionation. It was of interest to see the effect of different pH and the presence of neutral salt on the fractionation of normal meconium. 0.2 M acetate buffers of pH 3.75, 4.7, and 5.65* and 1 M phosphate buffers of pH 6.8 and 7.7* were prepared.

A sample of the original meconium suspension was diluted with water and centrifuged to remove the water insoluble material. Portions of the supernatant were treated as follows:

a. 4.5 cc. portions were treated with 0.5 cc. of each of the 0.2 M acetate buffer solutions, and 95% alcohol was gradually added to each solution in the cold room, 38° F. No appreciable precipitation occurred until an alcohol concentration of 57% was reached. After centrifugation, it was impossible to decant cleanly, but as much supernatant was removed as possible. Small, definite amounts of precipitate were separated at alcohol concentrations of 70 and 80%.

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* To prepare 0.2 M acetate buffer, pH 3.75, 1 cc. of 0.2 M sodium acetate was added to 9 cc. of 0.2 M acetic acid; 0.2 M acetate buffer, pH 4.7, 5 cc. of 0.2 M sodium acetate were added to 5 cc. of 0.2 M acetic acid; 0.2 M acetate buffer, pH 5.65, 1 cc. of 0.2 M acetic acid was added to 9 cc. of 0.2 M sodium acetate; 1 M phosphate buffer, pH 6.8, 1 cc. of 1 M $Na_2HPO_4 \cdot 12H_2O$ was mixed with 1 cc. of 1 M $NaH_2PO_4 \cdot H_2O$; and 1 M phosphate buffer, pH 7.7, 0.1 cc. of 1 M $NaH_2PO_4 \cdot H_2O$ was mixed with 0.9 cc. of 1 M $Na_2HPO_4 \cdot 12 H_2O$.

The pH 5.7 supernatant was made 83% with respect to alcohol, 0.5 cc. of 2 M acetate buffer, pH 5.7, and later 0.12 cc. of a 1 M solution of $Na_2HPO_4 \cdot 12H_2O$ were added. The alcohol concentration was high enough to cause the phosphate to crystallize, but there was a different type of precipitate present also.

Each precipitate was suspended in water. Samples of each supernatant and precipitate were analyzed for reducing sugar content.

b. 4.0 cc. portions of the supernatant were treated with 0.1 cc. of each of the 1 M phosphate buffer solutions. Absolute alcohol was gradually added to each solution in the cold room, 38° F. A little material separated at a 33% alcohol concentration. Precipitates also separated at alcohol concentrations of 50, 60, 67, 71, and 82%.

Each precipitate was suspended in water. Samples of each supernatant and precipitate were analyzed for reducing sugar content.

c. 4.6 cc. of the supernatant were treated with 0.42 cc. of 1.2 N HCl to make the total mixture about 0.1 N with respect to hydrochloric acid. This caused an immediate precipitation which formed a 'mucin clot' on centrifugation and was hard to separate by decanting.

Absolute alcohol was gradually added in the cold room, 38° F. Precipitates were separated at alcohol concentra-

tions of 53, 57, 64, 73, and 78%.

Each precipitate was suspended in water. Samples of each precipitate and the supernatant were analyzed for reducing sugar content.

Results:

Sample	Physical Description of Solution	Reducing Sugar mg.	% of Total
0.1 N HCl			
Original		24.0	----
I. HCl insoluble	Large amount of insoluble material, denatured (?)	3.6	15.0
II. 53% alcohol	Some insoluble material	1.1	4.6
III. 57% alcohol	Some insoluble material	5.1	21.2
IV. 64% alcohol	Cloudy solution	3.5	14.6
V. 73% alcohol	Cloudy, but homogeneous	5.3	22.1
VI. 78% alcohol	Clear solution	0.7	2.9
VII. Supernatant	Clear, green solution	4.4	18.3
Recovery		23.7	98.7
Acetate, pH 3.75			
Original		36.0	----
I. 57% alcohol	Homogeneous, cloudy, viscous	6.9	19.2
II. 70% alcohol	Homogeneous, opalescent	3.0	8.3
III. 80% alcohol	Homogeneous, clear, slightly viscous	3.6	10.0
IV. Supernatant	Cloudy	13.5	37.5
Recovery		27.0	75.0
Acetate, pH 4.7			
Original		36.0	----
I. 57% alcohol	Some insoluble material	11.5	31.9
II. 70% alcohol	Homogeneous, opalescent	4.1	11.4
III. 80% alcohol	Homogeneous, clear, viscous	6.4	17.8
IV. Supernatant	Cloudy	14.4	40.0
Recovery		36.4	101.1

Sample	Physical Description of Solution	Reducing Sugar mg.	% of Total
Acetate, pH 5.65			
Original		34	----
I. 57% alcohol	Some insoluble material	15.6	45.9
II. 70% alcohol	Fairly clear, viscous	4.1	12.1
III. 80% alcohol	Clear, viscous	12.5	36.8
IV. 83% alcohol	Clear	1.1	3.2
V. Supernatant	Clear	4.9	14.4
Recovery		38.2	112.4
Phosphate, pH 6.8			
Original		24.3	----
I. 33% alcohol	Some insoluble material	0.7	2.9
II. 50% alcohol	Some insoluble material	0.5	2.0
III. 60% alcohol	Some insoluble material	2.4	9.9
IV. 67% alcohol	Some insoluble material	3.0	12.3
V. 71% alcohol	Homogeneous, clear	2.6	10.7
VI. 82% alcohol	Homogeneous, clear, viscous	4.3	17.7
VII. Supernatant	Cloudy	14.5	59.7
Recovery		28.0	115.2
Phosphate, pH 7.7			
Original		24.3	----
I. 33% alcohol	Some insoluble material	0.8	3.3
II. 50% alcohol	Some insoluble material	0.5	2.0
III. 60% alcohol	Some insoluble material	1.6	6.6
IV. 67% alcohol	Some insoluble material	2.3	9.5
V. 71% alcohol	Homogeneous, clear	2.3	9.5
VI. 82% alcohol	Homogeneous, clear	4.4	18.1
VII. Supernatant	Cloudy	15.5	63.8
Recovery		27.4	112.8

Discussion: The fractionation with 0.1 N HCl gave indications of definite fractionations, but was probably quite drastic. The fact that some material precipitated with acid

alone and the early fractions failed to be water soluble may have been indications that the acid denatured or changed the material chemically.

The fractionation with acetate buffer at pH 3.75 did not show any sharp separation and was generally unsatisfactory.

At pH 4.7 there was a definite fraction with 57% alcohol, but a good deal of material remained in the final supernatant.

With acetate buffer at pH 5.65 there were definite fractions with 57% and with 80% alcohol. The material remaining in solution in the final supernatant probably corresponded to the soluble-dialyzable portion of normal meconium, although there was some indication that there had not been sufficient buffering or neutral salt present.

The phosphate fractionations were not satisfactory at all, possibly due to the insolubility of the phosphate in alcohol.

3. Third Fractionation.

a. Procedure. The fractionation with acetate buffer at pH 5.65 (see page 121) was repeated using 2 M instead of 0.2 M buffer solution.

Again a sample of the original meconium suspension was diluted with water and centrifuged to remove any water-insoluble material. 4.5 cc. of the supernatant were added to 0.5 cc. of the 2 M acetate buffer, pH 5.65. Absolute alcohol was gradually added to the mixture in the cold room, 38° F.

Precipitates were separated with 50, 60, 67, 75, and 83% alcohol concentrations. Further addition of alcohol did not cause more precipitation.

b. Characterization of the Products. Each precipitate was dissolved in water. Aliquots of each precipitate and the supernatant were analyzed for reducing sugar content. Results:

Sample	Physical Description of Solution	Reducing Sugar mg.	% of Total
Original		24.0	----
I. 50% alcohol	Very cloudy	3.5	14.6
II. 60% alcohol	Very cloudy	4.2	17.5
III. 67% alcohol	Fairly clear	2.3	9.6
IV. 75% alcohol	Clear and viscous	8.5	35.4
V. 83% alcohol	Clear	2.4	10.0
VI. Supernatant	Clear	4.1	17.1
Recovery		25.0	104.2

c. Characterization of Fraction IV.

1) 0.2 cc. of the fraction IV were treated with 0.05 cc. of 2 M acetate buffer, pH 5.65 and diluted to 1 cc. 3 cc. of absolute alcohol, added in the cold room, caused the mixture to become cloudy but did not cause the formation of any precipitate. When another cc. of absolute alcohol was added a definite precipitate formed. The precipitate and supernatant were analyzed for reducing sugar content.

Results:

Sample	Reducing Sugar mg.	% of Total
Original	1.302	----
Precipitate	1.270	97.5
Supernatant	0.075	5.8
Recovery	1.345	103.3

2) 0.2 cc. of fraction IV was treated with 0.05 cc. of 2 M acetate buffer, pH 5.65, and diluted to 1 cc. The addition of 6 cc. of saturated ammonium sulfate solution in the cold room caused only a slight cloudiness in the mixture. When it was saturated with solid ammonium sulfate, a little material formed but it did not represent a sizeable portion of the sample.

Discussion: The alcohol fractionation of normal meconium at pH 5.65 with acetate buffer appeared to be fairly reproducible and yielded definite fractions which could be decanted cleanly.

The largest fraction, which separated with approximately 75% alcohol, appeared quite homogeneous and was capable of being reprecipitated from solutions by a similar concentration of alcohol. Most of it was soluble in saturated ammonium sulfate solution.

4. Fourth Fractionation. A large amount of meconium was fractionated according to the same procedure described in the

third fractionation.

Aliquots of each precipitate and the supernatant were analyzed for reducing sugar content.

Results:

Sample	Reducing Sugar mg.	% of Total
Original	1,272.0	----
I. Water insoluble	40.5	3.2
II. 50% alcohol	43.4	3.4
III. 60% alcohol	296.3	23.3
IV. 67% alcohol	150.0	11.8
V. 75% alcohol	444.4	34.9
VI. 83% alcohol	97.5	7.7
VII. Supernatant	153.5	12.1
Recovery	1,225.6	96.4

a. First Purification. These fractions were purified as follows:

Fraction II, the 50% alcohol precipitate, contained some water insoluble material, so it was suspended in water and heavily centrifuged. The supernatant was treated with one-twentieth its volume of 2 M acetate buffer, pH 5.65, and then gradually with absolute alcohol in the cold room. A little material separated at an alcohol concentration of 44%, and definite precipitates at alcohol concentrations of 50 and 60%. The supernatant became cloudy at 67% but no precipitate formed until 75%.

Fraction III, the 60% alcohol fraction, formed a cloudy, viscous solution. It was treated with one-twentieth its

volume of 2 M acetate buffer, pH 5.65, and absolute alcohol was gradually added in the cold room. Precipitates separated at 50, 60, 67, and 75% alcohol.

Fraction IV, the 67% alcohol fraction, formed a fairly clear solution. It was treated with one-twentieth its volume of 2 M acetate buffer, pH 5.65, and absolute alcohol was gradually added in the cold room. A little precipitate separated at 50%, and larger fractions at 60, 67, and 75% alcohol.

Fraction V, the 75% alcohol fraction, formed a clear and very viscous solution. It was treated with one-twentieth its volume of 2 M acetate buffer, pH 5.65, and gradually with absolute alcohol. The solution became slightly cloudy with 50% alcohol concentration, but no precipitate formed until a 60% concentration was reached. Definite precipitates formed with 60, 67, 75, and 83% alcohol.

Fraction VI, the 83% alcohol precipitate, formed a clear solution. It was treated with one-twentieth its volume of 2 M acetate buffer, pH 5.65, and gradually with absolute alcohol. A little material separated at 67%, and more at 75 and 83% alcohol concentrations.

The 44% and 50% alcohol precipitates of fraction II and 50% precipitates of fractions III and IV were combined to form fraction Ia.

The 60% precipitates of fractions III, IV, and V were combined to form IIa, the 67% precipitates of III, IV, V, and VI to form IIIa, the 75% precipitates of II, III, IV, V, and VI to form IVa, and the 83% precipitates of V and VI to form Va.

Aliquots of each new fraction, Ia-Va, were analyzed for reducing sugar content.

Results:

Sample	Reducing Sugar mg.	% of Total
Original	1,185.0	-----
Ia. 50% alcohol	182.5	15.4
IIa. 60% alcohol	55.0	4.6
IIIa. 67% alcohol	158.8	13.4
IVa. 75% alcohol	495.0	41.8
Va. 83% alcohol	76.3	6.4
Recovery	967.6	81.6

b. Second Purification. These fractions were further purified as follows:

Fraction Ia, the 50% alcohol fraction, formed a very cloudy solution which did not look homogeneous. It was treated with one-twentieth its volume of 2 M acetate buffer, pH 5.65, and gradually increasing amounts of absolute alcohol. With a 46% alcohol concentration, a clotty precipitate formed which was difficult to decant from completely. Precipitates also formed at 50, 60 and 67% alcohol concentrations.

Fraction IIa, the 60% alcohol precipitates, formed a cloudy solution, which was treated similarly. A little material separated at 50% alcohol concentration, and larger precipitates at 60 and 67%, and a little at 75% alcohol concentration.

Fraction IIIa, the 67% alcohol precipitates, formed a solution that was only slightly cloudy. It was treated similarly, forming a little precipitate at 50%, more at 60 and 67%, and a little precipitate at 75% alcohol concentration.

Fraction IVa, the 75% alcohol precipitates, gave a clear, viscous solution which was treated as above. It gave a little insoluble material with 50% alcohol, and more at 60, 67, 75, and 83% alcohol.

Fraction Va, the 83% alcohol precipitates, gave a clear solution which was treated as described above. A little precipitate formed with 75% alcohol and more with 83% alcohol.

The 50% alcohol precipitates from fractions Ia, IIa, IIIa, and IVa were combined to form fraction IIb; the 60% alcohol precipitates from Ia, IIa, IIIa, and IVa to form IIIb; the 67% alcohol precipitates from Ia, IIa, IIIa, and IVa to form IVb; the 75% alcohol precipitates from IIa, IIIa, IVa, and Va to form Vb; and the 83% alcohol precipitates from IVa and Va to form VIb.

Aliquots of each fraction were analyzed for reducing sugar and nitrogen content, and also for blood group activity.

Fraction Vb was again dissolved in water and treated as above. A little precipitate formed with 67% alcohol and more with 75% alcohol. They were each dissolved in water and aliquots tested for reducing sugar and nitrogen content, and also for blood group activity.

Results:

Sample	Reducing Sugar mg.	Reducing Sugar % of Total	N mg.	N % of Total	Ratio R.S./N	Blood Group Activity* A	Blood Group Activity* B
Original	967.6	----	----	----	---	---	---
Ib. 46% Alc.	73.8	7.6	23.0	13.1	3.2	2.9	0.3
IIb. 50% Alc.	24.0	2.5	8.2	4.7	2.9	4.8	0.5
IIIb. 60% Alc.	128.8	13.3	26.8	15.3	4.8	2.6	0.3
IVb. 67% Alc.	168.8	17.4	34.2	19.5	4.9	0.3	0.3
Vb. 75% Alc.	392.5	40.6	73.5	41.9	5.3	---	---
VIb. 83% Alc.	63.1	6.5	9.6	5.5	6.6	2.5	2.5
Recovery	851.0	87.9	175.3	----	---	---	---
Original	392.5	----	73.5	----	5.3	---	---
Vb'. 67% Alc.	105.0	26.7	21.3	29.0	4.9	0.4	0.4
Vb'. 75% Alc.	290.0	73.9	55.0	74.8	5.3	0.6	0.6
Recovery	395.0	100.6	76.3	103.8	---	---	---

Discussion: This large scale fractionation of normal meconium with alcohol at pH 5.65 gave approximately the same results as the previous fractionations at that pH.

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* The approximate amount of reducing sugar, expressed in μg. present in the 0.1 cc. of solution tested, inhibiting iso-agglutination.

In no fraction was the carbohydrate portion separated from the nitrogen, and after repeated purification, the carbohydrate to nitrogen ratio rose as the alcohol concentration rose. All fractions possessed blood group activity.

These results indicated that there were several distinct fractions in normal meconium, and there was little separation of blood group activity in these fractions, although in a few fractions there appeared to be a slight enhancement of B activity.

Disregarding the water insoluble portion and the supernatant of the 83% alcohol precipitate, the remaining fractions after purification accounted for 66.9% of the carbohydrate and 45.1% of the nitrogen in the original starting material.

I. Partial Protein Characterization of Meconium Ileus.

1. Protein Tests on Crude Suspension. A suspension of five grams of meconium ileus in 50 cc. of water was used for these tests.

a. Heat Coagulation. When heated directly the crude suspension only showed a little sign of coagulation, but when a drop of acetic acid was added, changing the pH from neutral to about pH 4, distinct coagulation occurred.

b. Trichloroacetic Acid Precipitation. The suspension gave a very large amount of precipitate when a 20% solution of trichloroacetic acid was added, leaving a very light

colored, clear supernatant.

The supernatant from the heat coagulable protein gave a further precipitate with 20% trichloroacetic acid, indicating the probable presence of at least two different protein entities.

c. Ammonium Sulfate Precipitation. The meconium ileus suspension did not precipitate when it was made 50% concentrated in respect to ammonium sulfate, but some precipitate did form when the suspension was saturated with ammonium sulfate.

d. Other Protein Precipitants. The meconium ileus suspension also yielded a precipitate when it was treated with tannic acid and sulfosalicylic acid.

2. Carbohydrate Distribution in Meconium Ileus.

a. Water Soluble Carbohydrate. One cc. of the meconium ileus suspension was heavily centrifuged yielding 0.1 cc. of precipitate. An aliquot of the supernatant was analyzed for reducing sugar according to the method described on page 23.

b. Zinc Sulfate-Barium Hydroxide Soluble Carbohydrate. One cc. of the meconium ileus suspension was treated with 0.2 cc. zinc sulfate and 0.2 cc. barium hydroxide, see page 23, mixed, and centrifuged. An aliquot of the supernatant was analyzed for reducing sugar.

c. Heat Coagulable Carbohydrate. One cc. of the meconium ileus suspension was diluted with 2 cc. of water and treated with a few crystals of sodium acetate (the mixture was acid to bromocresol green indicator). This was heated for fifteen minutes on a boiling water bath, yielding a large amount of precipitate which was separated by centrifugation. An aliquot of the supernatant was analyzed for reducing sugar.

Results:

Sample	% Reducing Sugar in Supernatant*	% of Original in Supernatant
Original	2.3	----
Water soluble	2.0	87.0
$ZnSO_4$-$Ba(OH)_2$	1.6	69.6
Heat Coagulable	1.3	56.5

* Mg. reducing sugar in supernatant divided by mg. of meconium ileus in the sample of meconium ileus suspension.

Discussion: These results would indicate that a considerable portion of the carbohydrate was combined with the protein portion which was precipitated by heat or by zinc sulfate and barium hydroxide. It is possible that the amount of barium hydroxide and zinc sulfate added were insufficient to cause complete deproteinization.

3. The Water Soluble, Alcohol Insoluble Portion of Meconium Ileus. One sample of meconium ileus suspension was centrifuged to remove the water insoluble material.

The supernatant was treated with six volumes of absolute alcohol, yielding a precipitate. This was washed three times with absolute alcohol and dried under vacuum over sodium hydroxide.

The precipitate was treated in the following ways:

a. One sample was analyzed directly for its reducing sugar content.

b. Another sample was treated with water and heated on a boiling water bath for fifteen minutes. The insoluble material was then separated by centrifugation, and an aliquot of the supernatant analyzed for reducing sugar.

c. One sample was suspended in water and treated with 0.2 cc. of barium hydroxide and 0.2 cc. zinc sulfate, see page 23. The mixture was centrifuged and an aliquot of the supernatant analyzed for reducing sugar.

d. One sample was suspended in water. After standing for a half hour, it was centrifuged and an aliquot of the supernatant analyzed for reducing sugar.

e. Another sample was suspended in water and the insoluble material washed three times with water. The insoluble material was then analyzed for reducing sugar.

Results:

Sample	% Reducing Sugar*
Original	7.3
Heated	7.0
$Ba(OH)_2$-$ZnSO_4$	2.3
Water Soluble	6.7
Water Insoluble	1.1

* Mg. reducing sugar divided by the mg. of sample at the beginning.

Discussion: From these results, it would appear that most of the reducing sugar content was water soluble, with only a comparatively small portion remaining water insoluble. Treating the substance with heat did not remove any reducing sugar from solution and appeared to increase the amount in solution slightly. The effect might be due to a denaturation of the protein and a splitting of the carbohydrate portion from the protein.

The low percentage of reducing sugar in the original may possibly be because the determination was not done in the most accurate range of the reducing sugar method.

The barium hydroxide-zinc sulfate treatment removed a large portion of the reducing sugar content from the supernatant, thus implying that there was a protein-carbohydrate complex which had not been denatured by the alcohol and was water soluble.

Even though most of the carbohydrate was water soluble, there was a considerable amount of precipitate which did not

dissolve at all. It was very probable that the preparation which involved the use of a high concentration of alcohol at room temperature caused the denaturization of some of the protein.

Here again, as in the heat coaguable and trichloroacetic acid tests, was an indication that meconium ileus had at least two distinct protein components.

J. Ammonium Sulfate Fractionation of Meconium Ileus.

1. Preparation. A sample of the meconium ileus suspension, as described on page 133, was centrifuged heavily to remove any water insoluble material. Saturated ammonium sulfate solution was added in various portions over the period of several days. All of the solutions were kept at a temperature of approximately 38° F. in the cold room.

No significant precipitate formed until the ammonium sulfate concentration was increased to 67%. This precipitate was removed by centrifugation, and the ammonium sulfate concentration of the supernatant was increased to 75%. After standing several hours, a flocculent, cream-colored precipitate formed, leaving a very clear supernatant.

The supernatant was brought to saturation with solid ammonium sulfate. This did not cause any further precipitation.

The 67% ammonium sulfate precipitate was completely soluble in water but formed a very opalescent, greenish solution.

The 75% ammonium sulfate precipitate was completely soluble in water, forming a clear, pale yellow green solution.

These two solutions and the final supernatant were dialyzed against distilled water overnight.

2. Characterization of the Products. Appropriate samples of the non-dialyzable portions were analyzed for nitrogen and reducing sugar content, using the methods described on page 46 and page 23.

Results:

Sample	Nitrogen mg.	Reducing Sugar mg.	Ratio R.S./N	pH
Theoretical Starting Amount	30.3	18.3	0.60	6.67
I. 67% Precipitate	12.9	5.7	0.44	5.42
II. 75% Precipitate	4.2	0.43	0.10	5.10
III. Supernatant	3.1	---	----	----
Recovery	66.7%	33.5%		

3. Cross-checking of the Products. In order to check on the homogeneity of the various fractions, the following cross-checks were done:

a. The non-dialyzable portion of fraction I, the 67% ammonium sulfate precipitate, was made 60% saturated with ammonium sulfate. This caused the separation of a little

precipitate which appeared quite inhomogeneous, so it was discarded. The supernatant was then brought to 67% saturation, which caused a considerable amount of precipitation. After separating the precipitate by centrifugation, the supernatant was brought to 71% saturation which caused only a little more precipitation.

The 67% precipitate was redissolved in water and dialyzed against distilled water overnight. Appropriate samples were then analyzed for reducing sugar and nitrogen. Results:

Sample	Reducing Sugar mg.	Nitrogen mg.	Ratio R.S./N
Theoretical Starting Amount	5.4	12.2	0.44
Non-dialyzable Portion	4.24	8.28	0.51
Recovery	78.5%	67.9%	

b. The non-dialyzable portion of fraction II, the 75% ammonium sulfate precipitate, did not give any sign of precipitation when it was 50% saturated with ammonium sulfate. However a definite precipitate did form when the ammonium sulfate concentration was brought to 67% saturation. This left a practically clear supernatant which did not give any further sign of precipitation with addition of more ammonium sulfate.

The 67% precipitate was dissolved in water and dialyzed against distilled water overnight. Appropriate samples were then analyzed for reducing sugar and nitrogen.

Results:

Sample	Reducing Sugar mg.	Nitrogen mg.	Ratio R.S./N
Theoretical Starting Amount	0.4	3.9	0.10
Non-dialyzable portion	0.22	1.45	0.15
Recovery	55%	37.2%	

c. The non-dialyzable portion of fraction III, the ammonium sulfate saturated supernatant, was treated with two volumes of alcohol, but there was no indication of precipitation.

Discussion: The ammonium sulfate fractionation of meconium ileus indicated the following:

a. The protein components of meconium ileus behaved like albumins, since they were completely precipitated by ammonium sulfate between 50% saturation and complete saturation.

b. The original fractionation gave an indication of two fractions - one precipitating at 67% saturation with ammonium sulfate and the other at 75% saturation. When repeat precipitations were done to check the homogeneity of the fractions, most of the 67% fraction, I, was reprecipitated by 67%

ammonium sulfate saturation. A large fraction of II, the 75% fraction, was reprecipitated at 67% ammonium sulfate saturation.

Because the ammonium sulfate concentrations were not completely accurate, and considerable losses were incurred during the dialysis, both from handling and dialysis, there were undoubtedly slight errors in the percentage accounted for in the original fractionation and the percentage recovered in the repeat precipitations.

However, there was definite evidence for two distinct fractions. Fraction I had a reducing sugar to nitrogen ratio of 0.44. On repeat precipitation the ratio rose to 0.51, but was still in the same range. Fraction II had a ratio of 0.10 which rose to 0.15 on repeat precipitation. These ratios were so widely different and remained so nearly the same that they would indicate two distinct fractions.

c. The supernatant of the ammonium sulfate fractionation contained some nitrogen but no carbohydrate, so it possibly contained amino acids or polypeptides from possible protein breakdown which could not be precipitated by saturated ammonium sulfate or alcohol.

d. The pH values of the original suspension and of the two fractions after dialysis showed that although the meconium ileus was originally approximately neutral, the pH at the isoelectric point was slightly on the acid side, the

pH of the second fraction being somewhat lower than that of the first.

K. Dialysis of Meconium Ileus.

1. Procedure. Two cc. of the meconium ileus suspension, described on page 133, were dialyzed against 20 cc. of distilled water for sixteen hours.

A sample of the non-dialyzable portion was again dialyzed against distilled water for sixteen hours in order to determine if the dialyzable portion of meconium ileus was present in a definite concentration, or if the dialysis was a gradual transfer which came to an equilibrium.

2. Characterization of the Products. Appropriate aliquots were analyzed for reducing sugar content, according to the method described on page 23.

Results:

Sample	Reducing Sugar mg.	% of Total %
Theoretical Starting Amount	6.92	----
I. Non-dialyzable	3.58	51.7
II. Dialyzable	0.47	6.8
Recovery	4.05	58.5
Theoretical Starting Amount	1.8	----
I. Non-dialyzable, after repeat dialysis	1.4	77.7
II. Dialyzable	----	----

Discussion: About 15% of the reducing sugar content of meconium ileus was dialyzable. When dialysis of the non-dialyzable portion was repeated, there was no indication of further diffusion of reducing sugar, showing that the non-dialyzable fraction was a definite fraction which did not diffuse gradually or come to equilibrium with the dialyzing medium.

L. Alcohol Fractionation of Meconium Ileus.

1. Preparation. A sample of the meconium ileus suspension, described on 133, was centrifuged to remove any water insoluble material. The supernatant was treated gradually with 95% alcohol in the cold room, 38° F. At a concentration of 56% alcohol a considerable amount of precipitate was formed. Precipitates were also separated at alcohol concentrations of 62, 66.5, and 81%. Each precipitate was dissolved in water.

2. Characterization of Products. Each of the precipitates and the supernatant were analyzed for reducing sugar and nitrogen content according to the methods described on page 23 and page 46.

Results:

Sample	Reducing Sugar mg.	Reducing Sugar % of Total	N mg.	N % of Total	Ratio R.S./N
Original	17.0	----	28.0	----	0.607
I. 56% Alcohol	3.0	17.7	6.4	22.8	0.47
II. 62% Alcohol	3.1	18.2	5.2	18.6	0.60
III. 66.5% Alcohol	1.9	11.2	2.6	9.3	0.73
IV. 81% Alcohol	0.7	4.1	2.6	9.3	0.27
V. Supernatant	0.6	3.5	0.7	2.5	0.86
Recovery	9.3	54.7	17.5	62.5	----

A portion of the 56% alcohol precipitate was insoluble in water. It was separated by centrifugation in a high speed centrifuge and analyzed. It contained 0.3 mg. of reducing sugar and 0.8 mg. of nitrogen, or approximately 10% of the original fraction. It possibly consisted mainly of material which had not been removed in the original separation of water insoluble material or else it had been denatured during the course of the experiment.

Discussion: The alcohol fractionation of meconium ileus appeared to give fairly definite fractions. The largest amount of precipitate was formed with alcohol concentrations between 56 and 67%. The ratios of reducing sugar to nitrogen were all below 1.0 and appeared to increase as the per cent alcohol increased. Very little material was soluble in 81% alcohol or was precipitated by an alcohol concentration between 66.5 and 81%.

M. Enzyme Activity on Normal Meconium and Meconium Ileus.

Since the action of pepsin and trypsin on material passing down the gastro-intestinal tract undoubtedly has a great affect upon their final composition or breakdown products, it was of interest to test the action of these enzymes on normal meconium. It was of special interest to test their action on meconium ileus because of the classical theory that it was formed partially as a result of the lack of tryptic activity in the intestines.

1. Activity on Blood Group Substance A.

a. Peptic Digestion. A 0.1 g. sample of blood group substance A, fraction II, whose preparation will be discussed on page 200, was dissolved in 10 cc. of 0.01 N HCl, forming a clear solution. This was covered with 4 cc. of toluene, and 0.2 cc. of enzyme solution containing 195 µg. of pepsin (crystalline pepsin of porcine origin from Armour Laboratories) was added. This was mixed, stoppered, and placed in the incubator at 38° C. After 48 hours, another 0.4 cc. of enzyme solution was added. After another 96 hours, the excess toluene was removed and 54 cc. of absolute alcohol were added. The precipitate was separated by centrifugation and suspended in water. The water-insoluble material was separated and washed once with water. Six volumes of abolute alcohol were added to the combined super-

natants. The precipitate was again separated by centrifugation, drained, redissolved, and again precipitated with six volumes of absolute alcohol with the aid of a little sodium acetate. The precipitate was separated, drained, and dissolved in water.

Aliquots were analyzed for reducing sugar and nitrogen content, according to the methods described on page 23 and page 46, and blood group activity as described on page 61.

b. Tryptic Digestion. A 0.1 g. sample of blood group substance A was dissolved in 10 cc. of a 0.1 M phosphate buffer, pH 7.6*, forming a very cloudy solution. This was covered with 4 cc. of toluene, and 0.2 cc. of enzyme solution containing 251 µg. of trypsin** was added. This was mixed, stoppered, and placed in the incubator at 38° C. After 48 hours, another 0.4 cc. of enzyme solution was added. After another 96 hours, the mixture was treated like the peptic digest described above.

2. Activity on Alcohol Fractions of Blood Group Substance.

a. Peptic Digestion. 5 cc. of the purest alcohol fractions which separated at an alcohol concentration of 46, 50,

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* To prepare 0.1 M phosphate buffer, pH 7.6, 2.6 cc. of 0.1 M $NaH_2PO_4 \cdot H_2O$ were added to 17.4 cc. of 0.1 M $Na_2HPO_4 \cdot 12H_2O$.

** Crystalline trypsin of bovine origin containing not more than 50% magnesium sulfate from Armour Laboratories.

60, 67, 75, and 83%, as described on page 127, 4 cc. of water, 1 cc. of 0.1 N HCl, 4 cc. of toluene, and 0.2 cc. of enzyme solution containing 190 μg. of pepsin were added to each tube. These were then mixed, stoppered, and placed in the incubator at 38° C. After 96 hours another 0.2 cc. of enzyme solution was added to each tube. After another 120 hours, 50 cc. of absolute alcohol and several drops of sodium acetate solution were added to each tube. The precipitate was separated by centrifugation, drained, and dissolved in water. These solutions were centrifuged to remove any water insoluble material. This material was washed once with water. The supernatants were treated with absolute alcohol and hydrochloric acid and sodium acetate where needed. The precipitates were again separated by centrifugation and redissolved in water.

Aliquots of each were analyzed for reducing sugar and nitrogen content and blood group activity.

b. Tryptic Digestion. 5 cc. of the purest alcohol fractions which separated at an alcohol concentration of 46, 50, 60, 67, 75, and 83%, as described on page 127, 0.65 cc. of 0.2 M $NaH_2PO_4 \cdot H_2O$, 4.35 cc. of 0.2 M $Na_2HPO_4 \cdot 12H_2O$, 4 cc. of toluene, and 0.2 cc. of enzyme solution containing 240 μg. of trypsin were mixed together. They were stoppered and placed in the incubator at 38° C. After 96 hours another 0.2 cc. of enzyme solution was added to each tube. After

another 120 hours, 50 cc. of absolute alcohol and a drop of concentrated hydrochloric acid were added to each tube. The precipitate was separated by centrifugation, drained, and dissolved in water. The solutions were centrifuged to remove any water insoluble material. This material was washed once with water. The supernatants were treated with absolute alcohol and hydrochloric acid and sodium acetate where needed. The precipitates were again separated by centrifugation and redissolved in water.

Aliquots of each were analyzed for reducing sugar and nitrogen content and blood group activity.

c. Tryptic Digestion on Those Samples Already Treated with Pepsin. 0.1 cc. enzyme solution containing 90 μg. of trypsin; 0.32 cc. of 0.2 M $NaH_2PO4 \cdot H_2O$; 2.5 cc. of blood group substance solutions, see 2a above, and blood group substance A solution, see 1a above, after peptic digestion; and 2 cc. of toluene were mixed and allowed to digest in the incubator at 38° C. After 50 hours, another 0.1 cc. of enzyme solution was added to each tube. The digestion was stopped after another 46 hours by adding 30 cc. of absolute alcohol, 2 drops of sodium acetate solution, and one drop of hydrochloric acid to each tube. The precipitates were separated by centrifugation, drained, and redissolved in water. The solutions were treated with absolute alcohol, and the precipitates again separated and redissolved. These

solutions were centrifuged to remove any water insoluble material and the supernatants analyzed for reducing sugar and nitrogen content and blood group activity.

Results:

Sample	Enzyme Treatment	Reducing Sugar mg.	N mg.	Ratio R.S./N	Blood Group Activity* A	B
A	None	35.3	5.00	7.07	0.2	---
	Pepsin	27.1	3.84	7.07	0.07	---
	Trypsin	24.8	3.12	7.87	0.06	---
	Pepsin and Trypsin	7.85	1.10	7.03	0.3	---
46% Alcohol	None	14.8	4.6	3.2	2.9	0.3
	Pepsin	10.3	2.64	3.89	13	13
	Trypsin	8.92	2.45	3.64	11	1
	Pepsin and Trypsin	2.75	0.71	3.87	11	11
50% Alcohol	None	2.4	0.82	2.9	4.8	0.5
	Pepsin	1.26	0.29	4.37	16	16
	Trypsin	1.42	0.36	3.89	2	18
	Pepsin and Trypsin	0.25**	0.076	3.2	10	10
60% Alcohol	None	12.9	2.7	4.8	2.6	0.3
	Pepsin	8.57	1.55	5.54	11	11
	Trypsin	8.96	1.74	5.13	11	11
	Pepsin and Trypsin	2.44	0.40	6.16	10	10
67% Alcohol	None	16.9	3.42	4.9	0.3	0.3
	Pepsin	11.7	1.91	6.13	15	15
	Trypsin	12.0	2.30	5.22	1.5	15
	Pepsin and Trypsin	3.29	0.50	6.57	13	13
75% Alcohol	None	29.0	5.5	5.3	0.6	0.6
	Pepsin	23.84	3.41	6.99	3	30
	Trypsin	24.77	3.74	6.62	3	31
	Pepsin and Trypsin	6.75	0.87	7.76	27	27
83% Alcohol	None	12.6	1.92	6.6	2.5	2.5
	Pepsin	7.10	0.90	7.92	9	9
	Trypsin	8.79	1.13	7.76	11	110
	Pepsin and Trypsin	1.59	0.20	7.96	64	64

* The approximate amount of reducing sugar, expressed in µg., present in the 0.1 cc. of solution tested, inhibiting iso-agglutination.

** Questionable because of salt concentration.

Sample	Enzyme Treatment	Original Reducing Sugar mg.	Final Reducing Sugar mg.	% Recovery	Original Nitrogen mg.	Final Nitrogen mg.	% Recovery
A	Pepsin	35.3	27.1	76.8	5.00	3.84	76.8
	Trypsin	34.8	24.8	71.3	4.94	3.12	63.2
	Pepsin and Trypsin	8.50	7.85	92.4	1.20	1.10	91.7
46% Alcohol	Pepsin	14.8	10.3	69.6	4.6	2.64	57.4
	Trypsin	14.8	8.92	60.3	4.6	2.45	53.3
	Pepsin and Trypsin	3.23	2.75	85.1	0.83	0.71	85.5
50% Alcohol	Pepsin	2.4	1.26	52.5	0.82	0.29	35.4
	Trypsin	2.4	1.42	59.2	0.82	0.36	43.9
	Pepsin and Trypsin	0.39	0.25*	64.1	0.09	0.076	84.4
60% Alcohol	Pepsin	12.9	8.57	66.4	2.7	1.55	57.4
	Trypsin	12.9	8.96	69.5	2.7	1.74	64.4
	Pepsin and Trypsin	2.68	2.44	91.0	0.48	0.40	83.3
67% Alcohol	Pepsin	16.9	11.7	69.2	3.42	1.91	55.8
	Trypsin	16.9	12.0	71.0	3.42	2.30	67.3
	Pepsin and Trypsin	3.65	3.29	90.1	0.60	0.50	83.3
75% Alcohol	Pepsin	29.0	23.84	82.2	5.5	3.41	62.0
	Trypsin	29.0	24.77	85.4	5.5	3.74	68.0
	Pepsin and Trypsin	7.45	6.75	90.6	1.06	0.87	82.1
83% Alcohol	Pepsin	12.6	7.10	56.3	1.92	0.90	46.9
	Trypsin	12.6	8.79	69.8	1.92	1.13	58.9
	Pepsin and Trypsin	2.22	1.59	71.6	0.28	0.20	71.4

* Questionable because of salt concentration.

Discussion: At first it was attempted to follow the course of the digestion. However, the absorption at 280 mμ due to amino acids with a benzene ring which remained in solution after the blood group substance was precipitated by six volumes of alcohol was not satisfactory because of the presence of toluene. The precipitation also did not seem complete, because the nitrogen contents of the supernatants varied with each individual precipitation in spite of the care used in adjusting pH and salt concentrations.

The most satisfactory means of testing the material for enzyme action was to carefully precipitate the blood group substance with six volumes of absolute alcohol and remove any water insoluble products, such as denatured proteins, by centrifuging a solution of the precipitate, and determining the reducing sugar to nitrogen ratio and blood group activity in the alcohol insoluble, water soluble material.

These results showed that although the ratio was increased in most instances, each fraction still remained a distinct entity in comparison with the other fractions. Peptic activity was generally greater than tryptic, and a combination was even more effective.

At no stage was there any indication of complete digestion to the point that the blood group activity of the fraction was significantly diminished.

3. Activity on Meconium Ileus and Normal Meconium.

a. Preparation. Samples of the meconium ileus suspension, described on page 133, and the original meconium suspension, described on page 91, diluted were used.

For peptic digestion, 5 cc. of each suspension were treated with 0.1 cc. of 1 N hydrochloric acid, 4.9 cc. of water, 0.2 cc. of enzyme solution containing 204 µg. of pepsin, and 1.0 cc. of toluene.

For tryptic digestion, 5 cc. of each suspension were treated with 0.65 cc. of 0.2 M $NaH_2PO_4 \cdot H_2O$, 4.35 cc. of 0.2 M $Na_2HPO_4 \cdot 12H_2O$, 0.2 cc. of enzyme solution containing 205 µg. of trypsin, and 1.0 cc. of toluene.

They were incubated at 38° C. After 48 hours and again after another 72 hours, 0.2 cc. more of the appropriate enzyme solutions were added to each tube.

After a total of 192 hours they were removed from the incubator.

b. Characterization of the Products.

The original suspensions were analyzed for their total reducing sugar and nitrogen content. One cc. aliquots of each original suspension and of each digest were treated with 2.0 cc. of a 20% trichloroacetic acid solution, diluted to 5.0 cc., mixed, and centrifuged. Samples of each were analyzed for their reducing sugar and nitrogen contents.

Also 2.0 cc. aliquots of each digest were dialyzed against 20 cc. of distilled water for twenty-one hours. Samples of each of the non-dialyzable and dialyzable portions were tested for their reducing sugar and nitrogen contents.

Results:

Sample	Total N mg.	Non-Protein N mg.	% of Total	Total Reducing Sugar mg.	Non-Protein Reducing Sugar	% of Total
Meconium Ileus						
Original	4.28	0.27	6.3	2.79	----	----
Peptic Digest	----	2.75	64.2	----	----	----
Tryptic Digest	----	0.53	12.4	----	-----	----
Normal Meconium						
Original	2.43	1.30	53.5	10.89	9.54	87.6
Peptic Digest	----	1.53	63.0	----	6.36	58.4
Tryptic Digest	----	1.72	70.8	----	6.11	56.2

Sample	Nitrogen mg.	% of Total	Reducing Sugar mg.	% of Total
Meconium Ileus				
Original	4.28	----	2.79	----
Peptic Dialyzable	2.11	49.4	0.36	12.9
Peptic Non-dialyzable	1.78	41.6	2.14	76.6
Tryptic Dialyzable	0.43	10.1	----	----
Tryptic Dialyzable	3.36	78.5	2.25	80.7
Normal Meconium				
Original	2.43	----	10.89	----
Peptic Dialyzable	0.79	32.7	1.53	14.1
Peptic Non-dialyzable	1.65	67.8	7.49	68.8
Tryptic Dialyzable	0.66	27.0	1.46	13.4
Tryptic Non-dialyzable	1.78	73.3	7.71	70.8

Discussion: Normal meconium was slightly affected by both enzymes, a little more by trypsin than by pepsin. For some reason the reducing sugar content dropped. Possibly this was due to impurities in the enzymes.

Meconium ileus was affected to a large extent by pepsin and just slightly by trypsin. In neither case was any significant amount of the carbohydrate made soluble in trichloroacetic acid. This would indicate that the carbohydrate portion was still attached to protein or protein breakdown products which were still precipitable by trichloroacetic acid.

The results with dialysis largely confirmed these findings, except that a little carbohydrate was dialyzable in the peptic digest.

The large amount of activity with pepsin and the slight amount of tryptic activity was very surprising. One of the customary treatments for meconium ileus is to flush the intestinal tract with a solution of pancreatin in the belief that it would hasten the solution of the meconium. The indications from these results suggest that pepsin would be of much greater effect in the treatment.

Actually, although meconium ileus has a very gummy, hardened consistency, if it is well suspended in water, most of it will dissolve easily. Therefore the flushing of the intestine with water or saline is probably as effective as

any enzyme treatment would be in alleviating the condition of meconium ileus.

N. Conclusion.

1. Normal meconium contained a polysaccharide (which consisted of galactose, hexosamine, and methyl pentose) and very little protein, but considerable amounts of nitrogen that were not accounted for by the hexosamine nitrogen. Other workers (see Tables II and III, pages 19 and 20) have worked with similar products and found that most of this nitrogen was accounted for by α amino acids. The largest amount of normal meconium consisted of material which was capable of inhibiting isoagglutination. No method was found in which the carbohydrate and nitrogen components could be separated without destroying this activity, although it was possible to fractionate meconium into several fractions which had varying ratios of reducing sugar to nitrogen and amounts of blood group activity.

2. In contrast, meconium ileus consisted mainly of a protein which was firmly connected to a polysaccharide. It did possess blood group activity so it must have the same essential groupings exposed as blood group substances from other sources.

3. Between 10 and 15% of both the nitrogen and reducing sugar content of normal meconium was dialyzable. A small

amount of reducing sugar was dialyzable in meconium ileus.

4. One fraction of normal meconium precipitated from solution when it was 83% saturated with ammonium sulfate, and another fraction separated at complete saturation. The supernatant still contained a considerable portion.

In contrast meconium ileus yielded a large fraction at 67% saturation and a smaller one at 75%, but none at 83% or at complete saturation. The supernatant contained nitrogen but no reducing sugar so it probably consisted of amino acids or polypeptides from protein breakdown.

5. Normal meconium, when adequately buffered at pH 4.7, yielded a water insoluble fraction and fractions with 46, 50, 60, 67, 75, and 83% alcohol. Almost one-half of the material was present in the 75% alcohol fraction. This fraction had a reducing sugar to nitrogen ratio of 5.3 and was very active in inhibiting isoagglutination.

Meconium ileus yielded a water insoluble fraction and fractions with 56, 62, 66.5, and 81% alcohol. The 56 and 62% fractions contained the largest amount of material which had reducing sugar to nitrogen ratios of 0.47 and 0.60. Only minimal amounts of nitrogen and reducing sugar remained in solution after a 66.5% alcohol concentration had been reached.

6. The action of pepsin and trypsin on the alcohol fractions of normal meconium further confirmed the existence of distinct fractions which could not be changed by the enzymes into the same end product.

These enzymes were also active upon meconium ileus causing the liberation of some of the nitrogen, but in no case did the end products resemble any of the fractions of normal meconium, since the carbohydrate was still attached to the protein and could be precipitated by trichloroacetic acid.

7. In April, 1950, Morgan and his co-workers (87) published an article describing the preparation and properties of a homogeneous blood group A substance from human pseudomucinous ovarian cyst fluids. The fluid was first treated with trichloroacetic acid to remove the excess protein, and the lyophilized supernatant was extracted with 90% phenol. The material was then fractionally precipitated with alcohol between narrow limits, and this repeated several times to attain homogeneity. Fractionation from a solution of anhydrous ethylene glycol or formamide with alcohol then yielded a product which contained only one component when examined in a Tiselius electrophoresis apparatus and an ultra-centrifuge at pH 4 and 8. The immunochemical studies confirmed this as did the fractional solubility tests in 30% alcohol.

Since this was the purest human material reported in the literature, it was used as a basis for comparison.

	Blood Group Specific Substances			
	Morgan	$Ba(OH)_2$-$ZnSO_4$ (page 76)	Sevag III (page 92)	Phenol II (page 97)
Reducing Sugar %	57	32.1	42.6	43.9
Amino Sugar %	37	18.8	25.9	28.4
Methyl Pentose %	13.5 (18.0)*	4.5	5.8	6.0
Nitrogen %	5.72	4.3	6.0	5.1
Acetyl %	9.0	6.0	9.6	8.9
Ratio R.S./N	9.96	7.46	7.1	8.6
Ratio A.S./R.S.	0.649	0.585	0.608	0.647
Ratio M.P./R.S.	0.237 (0.316)	0.140	0.136	0.137
A Activity**	0.2	---	0.1	0.5
B Activity**	---	---	16	25

* The first figure was obtained from the yield of acetaldehyde liberated on oxidation with periodate after mild acid hydrolysis, and the second figure was obtained by Kabat et al. using the colorimetric method of Dische and Shettles.

** The approximate amount of material inhibiting isoagglutination, expressed in µg. in 0.1 cc. of solution tested.

This comparison would seem to indicate that these fractions probably contained extraneous nitrogen, which was present as an impurity. It was observed that purification always increased the percentage of carbohydrate and raised the reducing sugar to nitrogen ratios, so it could be ex-

pected that further purification would raise the carbohydrate content and the ratio further. Of course, it is possible that the blood group substances occurring in meconium are completely different complexes and cannot be compared.

The methyl pentose content was very low in comparison. Although the method seemed adequate with standards, it should be more carefully checked to make sure that it is accurate when used with blood group substances which contain large amounts of hexoses.

The methyl pentose percentages in these samples from meconium appear to be similar to the methyl pentose content found in substances of animal origin, see Table III, page 20.

V. The Blood-Group-Specific Substances in Meconium

A. Aim of the Investigation.

The presence of blood-group-specific substances in meconium in large amounts was of great interest. The greatest part of the work that has been done on blood-group-specific substances has been done on those of animal origin because there are so few sources of human substances where the concentration is high enough to allow them to be separated and purified in sufficient quantities. In meconium the specific substances were found not only in large amounts but almost completely free of impurities.

In order to determine if the blood-group-specific substance in meconium corresponded with the blood type of the individual baby, a group of babies was studied. The blood of each baby was typed, and a sample of its saliva tested to find if it secreted blood group substances in its body fluids and tissues. A suspension of the meconium was also tested to see the type of specific material it contained.

One of the classical theories of meconium formation is that it is the accumulation of fetal swallowing of amniotic fluid and products of endogenous formation. Amniotic fluid has also been shown to contain blood-group-specific substances. Putkonen (28) in 1930 stated that these substances corresponded to the blood type of the baby

and not of the mother; however, Kabat et al. (49) reported a case in 1947 in which the type of substance in the amniotic fluid was compared with the blood type of the mother and baby. The mother was type A and the baby type O, but the investigators found A specific materials in the amniotic fluid. In order to check these statements the amniotic fluid from a series of patients was collected. The blood and saliva from both the mother and baby and the meconium from the baby were also collected. In the cases where the mother was either blood type A, B, or AB and the baby had a different blood type than the mother, the saliva of each was studied to determine if they were secretors or non-secretors of blood-group-specific substances, and the amniotic fluid and meconium tested to see what type of blood-group-specific substance they contained. From this it was hoped to obtain a clue to whether meconium was formed partly from swallowed amniotic fluid or not.

Since meconium proved such a rich source of type A, B, AB, and O specific substances, it was also of interest to see if it contained other substances such as Rh, M, and N, especially since Witebsky (28) had reported finding Rh material in amniotic fluid.

Not only was it of interest to see what specific material the individual meconiums contained but also to determine the chemical composition of them, to see if there

were any gross differences between the different specific substances and also to see if they were comparable in composition to the analyses reported in the literature by other workers.

Sevag purifications were done on some of the samples of crude meconium and the chemical composition compared with that of the original meconium.

A sample of meconium from a calf and also from a dog were studied and compared with the samples of human meconium to see if that from other animals was similar chemically.

B. Comparison of the Blood, Saliva, and Meconium.

Samples of the blood, saliva, and meconium of a series of babies were collected. The blood was typed as described on page 53, the saliva collected and analyzed as described on page 60, and the meconium as described on page 61.

Results:

Baby's Name	Blood Type	Saliva Activity	Meconium Activity*	Classification
D. Thompson	A	A	$1:10^5$ A	A secretor
D. McDowell	A	A	$1:10^5$ A	"
M. Nutt	A	A	$1:10^5$ A	"
J. Kellum	A	A	$1:10^4$ A	"
B. Fetter	A	A	$1:10^5$ A	"
Weigel	A	A	$1:10^3$ A	"
H. Ross	A	A	$1:10^4$ A	"
M. Hollen	A	A	$1:10^4$ A	"
E. James	A	A	$1:10^4$ A	"
F. Parks	A	A	$1:10^5$ A	"
E. McRoberts	A	A	$1:10^5$ A	"
M. Hamilton	A	A	$1:10^7$ A	"
V. McWilliams	A	A	$1:10^7$ A	"
E. Beagle	A	A	$1:10^4$ A	"
E. Jones	A	A	$1:10^5$ A	"
P. Simpson	A	A	$1:10^5$ A	"
E. Johns	A	-	-	A non-secretor
V. Riley	A	-	-	"
V. Goodin	A	-	-	"
A. Collins	A	-	-	"
S. Wright	AB	A, B	$1:10^2$ A, $1:10^3$ B	AB secretor
M. Williams	AB	A, B	$1:10^3$ A, $1:10^3$ B	"

Baby's Name	Blood Type	Saliva Activity	Meconium Activity*	Classification
V. Boner	B	B	$1:10^4$ B	B secretor
W. Spradlin	B	B	$1:10^4$ B	"
L. Jones	B	B	$1:10^4$ B	"
H. Johnson	B	B	$1:10^7$ B	"
J. Williams	B	B	$1: >10^7$ B	"
B. Conners	B	B	$1:10^6$ B	"
H. Floyd	B	B	$1:10^6$ B	"
V. Hughbanks	B	-	-	B non-secretor
M. Coleman	B	-	-	"
C. Davis	B	-	-	"
M. Burns	O	O	$1:10^3$ O	O secretor
W. Barton	O	O	$1:3 \times 10^2$ O	"
I. Godbey	O	O	$1:10^2$ O	"
J. Lawrence	O	O	$1:10^3$ O	"
A. Leonard	O	O	$1:10^2$ O	"
P. Perkins	O	O	$1:3 \times 10^2$ O	"
L. Scott	O	O	$1:10^3$ O	"
J. Sherman	O	O	$1:3 \times 10^2$ O	"
S. Sloan	O	O	$1:3 \times 10^2$ O	"
M. Smedley	O	O	$1:10^2$ O	"
E. Ware	O	O	$1:10^3$ O	"
E. Ashley	O	-	-	O non-secretor
L. Ball	O	-	-	"
N. Binkley	O	-	-	"

Baby's Name	Blood Type	Saliva Activity	Meconium Activity*	Classification
O. Oliver	O	-	-	O non-secretor
M. Parker	O	-	-	"
S. Russell	O	-	-	"
D. Terrill	O	-	-	"
E. Whitaker	O	-	-	"
R. Dubky	O	-	$1:3 \times 10^2$ O	?
R. Howard	O	-	$1:10^2$ O	?

* The meconium was originally suspended in 3 times its weight of water. These figures represent the approximate dilution of the suspensions at which agglutination first appeared.

Classification	Number	Per cent	
A secretors	16	22.9	28.6
A non-secretors	4	5.7	
AB secretors	2	2.8	2.8
B secretors	7	10.0	14.3
B non-secretors	3	4.3	
O secretors	11	----	54.3
O non-secretors	8	----	
O ?	2	----	
O not analyzed	17	----	
Total	70	----	100.0

Discussion: In all cases except two the meconium possessed blood group activity if the saliva of that baby showed that it possessed the ability to secrete blood group substances in its body fluids and tissues. In all instances the activity found in the meconium corresponded with the blood type of the baby. The A and B determinations were all very clean cut and definite, except for the two babies of type AB. The agglutinations of the blood were slow forming with anti-A serum, so possibly they belonged to a subgroup of A. This could explain the low amount of blood group activity in the meconium of these individuals.

The O activity was much lower in all cases. Probably this was the result of the weak antigen activity of the blood type O specific substance. Generally any indication of O activity in the saliva was accompanied by at least some activity in the meconium.

In two cases the meconium possessed O activity, but there was no indication of activity in the saliva. It is possible that the substance was present in such small amounts in the saliva that the tests as used were not sufficiently sensitive to show it.

Because the meconium had proved such a rich source of the four main blood types, A, B, AB, and O, it was tested to see if it also contained Rh, and M and N activity.

The blood samples of six babies were tested for M and N activity as described on page 53. The meconium was then carefully tested for M and N activity. Also samples of the original meconium suspension, described on page 91, the Sevag fractions, described on page 92, and the phenol fractions, described on page 97, were all tested for M and N activity. In no case was any activity indicated.

These negative results correspond with the fact that M and N antigens appear to be restricted to the erythrocytes in normal individuals (25).

The crude suspensions and Sevag purifications of the meconium from Infants Allen, Hughbanks, McDowell, and Thompson and the Sevag purification of the meconium from Infant Boner, who were all Rh_o+, and the crude suspension and Sevag purification of the meconium of Infant Wilder, who was Rh_o- (cde), were tested for Rh_o+ activity. The following results were obtained, using the method described on page 61.

Sample	Meconium Dilution*	Tube Serum Dilution	Agglutination
Allen Meconium Rh_o+	$1:4.8 \times 10^3$	1:8	-
	$1:4.8 \times 10^4$	1:8	+++
	$1:4.8 \times 10^3$	1:16	-
	$1:4.8 \times 10^4$	1:16	tr
Allen Sevag Rh_o+	1:100	1:8	+++
	1:1000	1:8	+++
	1:100	1:16	+
	1:1000	1:16	+
Boner Sevag Rh_o+	1:100	1:8	++
	1:1000	1:8	++++
	1:100	1:16	++
	1:1000	1:16	++
Hughbanks Meconium Rh_o+	$1:5.6 \times 10^3$	1:8	-
	$1:5.6 \times 10^4$	1:8	+++
	$1:5.6 \times 10^3$	1:16	-
	$1:5.6 \times 10^4$	1:16	tr
Hughbanks Sevag Rh_o+	1:100	1:8	++++
	1:1000	1:8	++++
	1:100	1:16	+
	1:1000	1:16	++
McDowell Meconium Rh_o+	$1:1.6 \times 10^3$	1:8	±
	$1:1.6 \times 10^4$	1:8	++++
	$1:1.6 \times 10^3$	1:16	+
	$1:1.6 \times 10^4$	1:16	+
McDowell Sevag Rh_o+	1:100	1:8	++
	1:1000	1:8	+++
	1:100	1:16	+
	1:1000	1:16	++
Thompson Meconium Rh_o+	$1:4.8 \times 10^3$	1:8	-
	$1:4.8 \times 10^4$	1:8	++++
	$1:4.8 \times 10^3$	1:16	-
	$1:4.8 \times 10^4$	1:16	+
Thompson Sevag Rh_o+	1:100	1:8	++++
	1:1000	1:8	+++
	1:100	1:16	++++
	1:1000	1:16	++

Sample	Meconium Dilution*	Tube Serum Dilution	Agglutination
Wilder Meconium Rh_o-	1:6.1 x 10^{-3}	1:8	-
	1:6.1 x 10^{-4}	1:8	+++
	1:6.1 x 10^{-3}	1:16	-
	1:6.1 x 10^{-4}	1:16	++
Wilder Sevag Rh_o-	1:100	1:8	++++
	1:1000	1:8	++++
	1:100	1:16	+
	1:1000	1:16	+

* Calculated from the dry weight of the material.

The crude meconium of Infants Allen, Hughbanks, Thompson, and Wilder showed definite inhibition of agglutination while the McDowell sample showed only slight inhibition. All of the Sevag purified products showed considerably less activity, indicating that it was destroyed or removed during purification.

No explanation could be made for the presence of Rh_o+ material in the Wilder sample. The blood type of the mother was not obtained, so it was not known whether she was Rh_o+. Witebsky (28) had stated that in several cases the amniotic fluid contained Rh_o+ material when the baby was Rh_o-, and could give no explanation for its presence.

This finding requires elaboration and more detailed work since it could possibly be of importance in the understanding of erythroblastosis fetalis.

C. Comparison of Amniotic Fluid with Meconium

Samples of amniotic fluid, the blood and saliva of both the mother and the baby, and the meconium were collected. The samples of blood were typed according to the method described on page 53. . Out of a series of thirty deliveries, five cases were found in which the mother had either A, B, or AB blood type and the baby had a different blood type. The salivas were then tested to see if they were secretors of A or B substances, and the amniotic fluid and meconium tested to see what type of activity was present. The methods used are described on page 60 and page 61.

Results: Name	Blood of Mother	Saliva of Mother	Blood of Baby	Saliva of Baby	Amniotic Fluid Activity	Meconium Activity
Schoolie	AB	Weak AB Secretor	B	B Secretor	B	B
Martin*	A	Non-secretor	O	----	No A	No A
Shelton	B	B Secretor	O	----	No B	No B
Hicks*	B	B Secretor	O	----	No B	No B
Williams*	B	B Secretor	O	----	No B	No B

* Artificially collected by a syringe from unbroken membranes.

Discussion: This experiment indicated that the blood group substance in amniotic fluid was of fetal origin. The exact source of the amniotic fluid is a matter of controversy. Some people believe it to be mainly an accumulation of fetal

urine, while some have indicated that they believe it is a transudate of maternal blood (88).

These results were in contradiction to the statement of Kabat, et al. (49) that they had obtained amniotic fluid containing A specific substance from a mother who had A blood type and a baby who had O blood type. Their sample must have been greatly contaminated because no activity was found in either the Schoolie or Shelton samples which had not been collected artificially and could have contained some vaginal mucus from the mother.

The results confirm the findings of Witebsky (28) and Putkonen who had found the specific substance to correspond with the blood type of the baby.

The methods used were satisfactory for several other samples of amniotic fluid tested. From these tests amniotic fluid appeared to contain approximately the same amount of blood-group-specific substance as saliva.

This experiment did not give any indication for the source of the group specific material that was found both in it and meconium, except that since they both corresponded to the blood type of the baby, the ultimate origin was fetal.

The theory that meconium is the result of fetal swallowing of amniotic fluid was not disproved.

D. Chemical Comparison of Individual Meconiums.

From the large group of individual meconium samples collected a few representatives of each of the blood types, A, AB, B, and O, were selected and tested in greater detail. Also a sample of dog meconium was collected from the entire intestine of several puppies which died shortly after birth. A calf's intestines were received through the courtesy of Louis B. Kahn of Cincinnati and the meconium removed from the large intestine, colon, and rectum. These samples were tested in the same way as the human meconium samples in order to see if meconium from other mammals compared chemically with that of humans.

Portions of some of the meconium samples were subjected to Sevag purification, according to the method as described on page 92. These purified substances were then compared chemically with the crude meconium samples.

The Sevag purification was repeated on several of the samples in order to get an indication of the reproducibility of the method.

Reducing sugar determinations were done as described on page 23, amino sugar determinations as described on page 26, methyl pentose determinations as described on page 28, nitrogen content as described on page 46, acetyl content as described on page 51, and A and B blood group activity as described on page 61.

Sample	Blood Type**	Reducing Sugar % *	Amino Sugar % *	Methyl Pentose % *	Nitrogen % *	Ratio A.S./ R.S.	Ratio M.P./ R.S.	Ratio R.S./N
McDowell Meconium	A S	30.7	18.7	5.3	7.2	0.61	0.17	4.26
McDowell Sevag	A S	37.3	23.2	7.7	6.6	0.62	0.21	5.65
Fetter Meconium	A S	25.6	14.1	4.2	6.6	0.55	0.16	3.88
Kellum Meconium	A S	30.2	16.1	4.5	6.5	0.53	0.15	4.65
Thompson Meconium	A S	31.5	18.3	5.0	6.8	0.58	0.16	4.63
Thompson Sevag	A S	36.0	23.0	---	5.7	0.64	----	6.32
Weigel Meconium	A S	29.0	17.0	4.3	5.9	0.59	0.15	4.92
Boner Meconium	B S	26.2	15.5	3.6	6.8	0.59	0.14	3.85
Boner Sevag	B S	37.0	22.0	6.0	6.7	0.59	0.16	5.52
Spradlin Meconium	B S	26.7	15.1	5.2	5.9	0.57	0.20	4.53
Spradlin Sevag	B S	36.0	23.0	---	6.4	0.64	----	5.62
Hughbanks Meconium	B s	28.6	16.3	3.0	6.2	0.57	0.11	4.61
Hughbanks Sevag	B s	36.0	22.4	---	6.4	0.62	----	5.62
Wright Meconium	AB S	27.4	16.3	5.9	6.1	0.59	0.21	4.49
Wright Sevag	AB S	41.6	24.8	8.4	6.2	0.60	0.21	6.71
Williams Meconium	AB S	31.4	17.2	5.4	6.4	0.55	0.17	4.91
Wilder Meconium	O	27.6	16.9	3.2	5.9	0.61	0.12	4.68
Wilder Sevag	O	40.0	23.0	5.2	6.7	0.57	0.13	5.97
Allen Meconium	O	30.5	19.7	3.4	7.3	0.65	0.11	4.18
Allen Sevag	O	34.0	24.5	---	6.3	0.72	----	5.40
Calf Meconium	-	12.0	6.8	1.0	6.2	0.57	0.08	1.93
Calf Sevag	-	36.6	22.8	4.6	5.4	0.62	0.13	6.78
Dog Meconium	-	5.6	4.1	1.4	9.7	0.73	0.19	0.58
Dog Sevag	-	14.1	9.8	---	7.8	0.70	----	1.81

* On the dry weight basis.
** S means that blood group substance was secreted in the saliva; s means that no blood group substance was secreted in the saliva.

Sample	Blood Type*	Total N % *	Amino Sugar % *	Amino Sugar N*** % *	Ratio A.S.N/ Total N	N Calcd. as Protein N**** % *	Calcd. as Protein %
McDowell Meconium	A S	7.2	18.7	1.46	0.21	5.74	35.9
McDowell Sevag	A S	6.6	23.2	1.81	0.27	4.79	29.9
Fetter Meconium	A S	6.6	14.1	1.10	0.17	5.50	34.4
Kellum Meconium	A S	6.5	16.1	1.26	0.20	5.24	32.8
Thompson Meconium	A S	6.8	18.3	1.43	0.21	5.37	33.6
Thompson Sevag	A S	5.7	23.0	1.79	0.31	3.91	24.4
Weigel Meconium	A S	5.9	17.0	1.33	0.22	4.57	28.6
Boner Meconium	B S	6.8	15.5	1.21	0.18	5.59	34.9
Boner Sevag	B S	6.7	22.0	1.72	0.25	4.98	31.1
Spradlin Meconium	B S	5.9	15.1	1.18	0.20	4.72	29.5
Spradlin Sevag	B S	6.4	23.0	1.79	0.28	4.61	28.8
Hughbanks Meconium	B s	6.2	16.3	1.27	0.21	4.93	30.8
Hughbanks Sevag	B s	6.4	22.4	1.75	0.27	4.65	29.1
Wright Meconium	AB S	6.1	16.3	1.27	0.21	4.83	30.2
Wright Sevag	AB S	6.2	24.8	1.94	0.31	4.26	26.6
Williams Meconium	AB S	6.4	17.2	1.34	0.20	5.06	31.6
Wilder Meconium	O	5.9	16.9	1.32	0.22	4.58	29.2
Wilder Sevag	O	6.7	23.0	1.80	0.27	4.90	30.6
Allen Meconium	O	7.3	19.7	1.54	0.21	5.76	36.0
Allen Sevag	O	6.3	24.5	1.91	0.30	4.39	27.4
Calf Meconium	-	6.2	6.8	0.53	0.08	5.67	35.4
Calf Sevag	-	5.4	22.8	1.78	0.33	3.62	22.6
Dog Meconium	-	9.7	4.1	0.32	0.033	9.38	58.8
Dog Sevag	-	7.8	9.8	0.76	0.097	7.04	43.8

* On the dry weight basis.

** S means that blood group substance was secreted in the saliva; s means that no blood group substance was secreted in the saliva.

*** Based on the assumption that nitrogen is 7.8% of the amino sugar.

**** Total nitrogen minus the amino sugar nitrogen. This percentage times the protein factor 6.25, see page 67, gives the approximate amount of protein.

Sample	Blood Type**	Reducing Sugar %*	Acetyl %*	Calcd. as Protein %*	Total %*
McDowell Meconium	A S	30.7	---	35.9	66.6
McDowell Sevag	A S	37.3	8.2	29.9	75.4
Fetter Meconium	A S	25.6	---	34.4	60.0
Kellum Meconium	A S	30.2	---	32.8	63.0
Thompson Meconium	A S	31.5	---	33.6	65.1
Thompson Sevag	A S	36.0	---	24.4	60.4
Weigel Meconium	A S	29.0	---	28.6	57.6
Boner Meconium	B S	26.2	---	34.9	61.1
Boner Sevag	B S	37.0	6.6	31.1	74.7
Spradlin Meconium	B S	26.7	---	29.5	56.2
Spradlin Sevag	B S	36.0	---	28.8	64.8
Hughbanks Meconium	B s	28.6	---	30.8	59.4
Hughbanks Sevag	B s	36.0	---	29.1	65.1
Wright Meconium	AB S	27.4	---	30.2	57.6
Wright Sevag	AB S	41.6	7.4	26.6	75.6
Williams Meconium	AB S	31.4	---	31.6	63.0
Wilder Meconium	O	27.6	---	29.2	56.8
Wilder Sevag	O	40.0	7.5	30.6	78.1
Allen Meconium	O	30.5	---	36.0	66.5
Allen Sevag	O	34.0	---	27.4	61.4
Calf Meconium	-	12.0	---	35.4	47.4
Calf Sevag	-	36.6	8.0	22.6	67.2
Dog Meconium	-	5.6	---	58.8	64.4
Dog Sevag	-	14.1	---	43.8	57.9

* On the dry weight basis.

** S means that blood group substance was secreted in the saliva; s means that no blood group substance was secreted in the saliva.

Protein values were calculated from nitrogen not otherwise accounted for and may not represent actual protein.

Sample	Blood Type**	Amino Sugar %*	Amino Sugar Mol %	Acetyl %*	Acetyl Mol %	Ratio mol % Acetyl/mol % Amino Sugar
McDowell Sevag	A S	23.2	0.130	8.2	0.191	1.47
Boner Sevag	B S	22.0	0.123	6.6	0.153	1.24
Wright Sevag	AB S	24.8	0.139	7.4	0.172	1.24
Wilder Sevag	O	23.0	0.128	7.5	0.174	1.36
Calf Sevag	-	22.8	0.127	8.0	0.186	1.46

* On the dry weight basis.

** S means that blood group substance was secreted in the saliva.

Sample	Blood Type*	Blood Group Activity** A	B
McDowell Meconium	A S	0.06	----
McDowell Sevag	A S	0.01	----
Fetter Meconium	A S	0.08	----
Kellum Meconium	A S	0.6	----
Thompson Meconium	A S	0.05	----
Thompson Sevag	A S	1.0	----
Weigel Meconium	A S	0.7	----
Boner Meconium	B S	----	0.7
Boner Sevag	B S	----	0.01
Spradlin Meconium	B S	----	0.7
Spradlin Sevag	B S	----	0.1
Hughbanks Meconium	B s	----	----
Hughbanks Sevag	B s	----	----
Wright Meconium	AB S	73	7.3
Wright Sevag	AB S	----	0.2
Williams Meconium	AB S	60	60
Wilder Meconium	O	----	----
Wilder Sevag	O	100?	----
Allen Meconium	O	----	----
Allen Sevag	O	----	----
Calf Meconium	-	----	----
Calf Sevag	-	----	----
Dog Meconium	-	----	----
Dog Sevag	-	----	----

* S means that blood group substance was secreted in the saliva; s means that no blood group substance was secreted in the saliva.

** The approximate amount of material inhibiting isoagglutination, expressed in μg. in 0.1 cc. of solution tested. The dashes indicate lack of activity with the highest amount tested, usually 1000 μg.

Sample		Blood Type**	Reducing Sugar %*	Amino Sugar %*	Nitrogen %*	Ratio A.S./R.S.	Ratio R.S./N	Blood Group Activity*** A	Blood Group Activity*** B
McDowell Sevag	I	A S	37.3	23.2	6.6	0.62	5.65	0.01	----
	II		38.0	24.5	7.2	0.64	5.28	1.0	----
Boner Sevag	I	B S	37.0	22.0	6.7	0.59	5.52	----	0.01
	II		36.0	23.0	7.1	0.64	5.07	----	0.1
Wright Sevag	I	AB S	41.6	24.8	6.2	0.60	6.71	----	0.2
	II		36.0	20.0	6.0	0.56	6.00	----	1.0
Wilder Sevag	I	O	40.0	23.0	6.7	0.57	5.97	100?	----
	II		36.0	19.4	5.9	0.54	6.10	----	----
Calf Sevag	I	-	36.6	22.8	5.4	0.62	6.78	----	----
	II		32.0	22.4	6.4	0.70	5.00	----	----

* On the dry weight basis.
** S means that blood group substance was secreted in the saliva.
*** The approximate amount of material inhibiting isoagglutination, expressed in μg. in 0.1 cc. of solution tested. The dashes indicate lack of activity with the highest amount tested, usually 1000 μg.

Sample		Blood Type**	Total N % *	Amino Sugar % *	Amino Sugar N*** % *	Ratio A.S.N/ Total N	N Calcd. as Protein N**** % *	Calcd. as Protein %
McDowell Sevag	I	A S	6.6	23.2	1.81	0.27	4.79	29.9
	II		7.2	24.5	1.91	0.27	5.29	33.1
Boner Sevag	I	B S	6.7	22.0	1.72	0.25	4.98	31.1
	II		7.1	23.0	1.79	0.25	5.31	33.2
Wright Sevag	I	AB S	6.2	24.8	1.94	0.31	4.26	26.6
	II		6.0	20.0	1.56	0.26	4.44	27.8
Wilder Sevag	I	O	6.7	23.0	1.80	0.27	4.90	30.6
	II		5.9	19.4	1.51	0.26	4.39	27.4
Calf Sevag	I	-	5.4	22.8	1.78	0.33	3.62	22.6
	II		6.4	22.4	1.75	0.27	4.65	29.1

* On the dry weight basis.
** S means that blood group substance was secreted in the saliva.
*** Based on the assumption that nitrogen is 7.8% of the amino sugar.
**** Total nitrogen minus the amino sugar nitrogen. This percentage times the protein factor 6.25, see page 67, gives the approximate amount of protein, assuming that all of the nitrogen other than that of the amino sugar was in the form of protein. This is an assumption only, since direct determinations of protein, as such, were not made. Other nitrogen compounds may have been present.

Sample	Reducing Sugar %*	Protein %*	Total %*
McDowell Sevag I	37.3	29.9	67.2
II	38.0	33.1	71.1
Boner Sevag I	37.0	31.1	68.1
II	36.0	33.2	69.2
Wright Sevag I	41.6	26.6	68.2
II	36.0	27.8	63.8
Wilder Sevag I	40.0	30.6	70.6
II	36.0	27.4	63.4
Calf Sevag I	36.6	22.6	59.2
II	32.0	29.1	61.1

* On the dry weight basis.
The values for protein were calculated from nitrogen not otherwise accounted for, and may therefore be too high.

Discussion: These results indicated the following:

a. All of the samples of meconium contained approximately the same amount of reducing sugar, amino sugar, methyl pentose, and nitrogen.

b. The Sevag purification increased the percentage of the sugars and therefore the reducing sugar to nitrogen ratio increased.

c. The Sevag purification also increased the methyl pentose percentage.

d. In all cases the methyl pentose to reducing sugar content ratio rose considerably upon Sevag purification.

e. The methyl pentose to reducing sugar ratios gave indications that they might be characteristic of each blood type:

	Blood Type			
	A	B	AB	O
Before Sevag Purification	0.173 0.164 0.149 0.159 0.148	0.137 0.195 (0.105)*	0.215 0.172	0.111 0.116
After Sevag Purification	0.206	0.162	0.207	0.130

* B non-secretor.

These figures indicated that the A and B meconium ratios were about the same, the AB ratios somewhat higher, and the O ratios lower. Although there were not enough samples for definite conclusions to be drawn, these at least were the only distinctions between groups that seemed very apparent from the carbohydrate determinations.

Those samples which possessed known A or B activity had a decidedly higher ratio than either the O meconiums or the B non-secretor meconium. This might be an indication that the amount of methyl pentose combined with the carbohydrate residue determined if the material would possess blood-group-specific activity.

f. The amino sugar nitrogen to total nitrogen ratio always increased with Sevag purification.

g. An attempt was made to determine the optical rotation on the Sevag purified products. However all of the solutions were so opalescent that accurate rotations could not be obtained.

h. The dog meconium had a very low carbohydrate percentage. This may be because the samples were collected from the entire intestine rather than the colon and rectum. However, even after Sevag purification the percentage was low. It also did not completely dissolve in water as the other Sevag preparations did.

i. Sevag purification did not appear to increase the effective blood group activity per unit weight; however no attempt was made to interpolate the activity titrations between ten-fold dilutions.

j. The A activity possessed by the Wilder meconium after Sevag purification was evidently due to impurities for the repeat purification did not possess any activity.

The blood cells of Wright were only weakly agglutinated by anti-A serum but strongly by anti-B serum. The crude meconium showed A group activity in amounts of 73 μg. but B activity with 7.3 μg. On purification the A activity disappeared, so possibly it represented a subgroup of A.

k. The Sevag purifications seemed to be fairly reproducible.

E. Paper Chromatography of Individual Meconiums.

In order to have a more definite indication of the individual reducing monosaccharides which composed the blood-group-specific substances and to see if there were any gross differences between the blood-group-specific substances of the different blood types, the paper chromatography of sugars method of Partridge (89,90) was used.

The polysaccharide to be analyzed was hydrolyzed into its component monosaccharides. A small amount of this solution was then placed in the form of a circular spot about a centimeter in diameter at the top of a strip of filter paper. The paper was hung vertically from a trough, which contained a solvent previously saturated with water, so that the top edge of the paper was immersed in the solvent. The trough and filter paper were then placed in a closed vessel which was kept in a condition of saturation both in respect to water and to the solvent. A sharp horizontal liquid front formed which advanced down the paper at a speed which depended upon the physical properties of the solvent.

Partition of the solute took place between the water bound by the cellulose and the solvent which moved over the surface of the cellulose fibers. The distance which a solute moved in the direction of flow was then a function of the partition coefficient of the solute between the water and

the wet solvent, the volume of water bound by a unit area of the filter paper, and the volume of wet solvent held by a unit area of the filter paper after irrigation. The volume of water and solvent held by the filter paper is substantially constant in a single experiment if the filter paper is uniform in texture, thickness, and water content. Therefore under ideal conditions the relative distance (R_f) moved by each solute is dependent upon its partition coefficient only.

Consden *et al.* defined R_f as the ratio between the distance moved by the solute and the distance moved by the advancing front of liquid.

To show the position of the spots formed by the reducing sugars, a solution containing silver nitrate and a large excess of ammonia was sprayed on the filter paper after the solvents used to irrigate it had been removed. When heated, metallic silver was precipitated in the region occupied by reducing sugars giving rise to brownish spots. The ammonia-silver nitrate reagent was effective for all reducing sugars.

If it was desired to show amino sugars specifically, the paper was sprayed first with an acetylacetone reagent and then with a modified Ehrlich reagent which formed red spots with the amino sugars according to the reaction described on page 26.

Procedure:

1. Preparation of the Blood Group Substances. Approximately 30 mg. of each of the Boner, Hughbanks, McDowell, Wilder, Wright, and calf blood group substances which had been purified by the Sevag method, see page 174, were treated with 1.5 cc. of 0.5 N sulfuric acid and hydrolyzed under reflux on a boiling water bath for four hours. An equivalent amount of barium hydroxide was added to each. The barium sulfate was removed and washed four times with 2.5 cc. of water. The combined supernatants all had a pH close to 5. The volume of the supernatants was reduced by lyophilization to 0.5 to 2.0 cc. These concentrated solutions were used for the chromatography.

2. Solvents. Three different solvent mixtures were used to get adequate separation of the monosaccharides.

a. Phenol (Merck crystallized) was saturated with water. Since amino sugars were expected in these materials, it was necessary to carry out the irrigation in an atmosphere containing ammonia, because they gave rise to elliptical spots or trails otherwise. Enough ammonia was therefore added to the liquid phenol to make it 1% with respect to ammonia. A few crystals of KCN were also added to the aqueous portion, since catalytic oxidation processes take place under the influence of traces of copper. In the absence of a small concentration of HCN, the advancing phenol front would

be dark with oxidation products.

b. s-Collidine was saturated with water.

c. Forty cc. of butyl alcohol, 10 cc. of glacial acetic acid, and 50 cc. of water were mixed, giving two distinct phases. Because it was a three-phase system, the volume of water was important.

3. Spray Solutions.

a. The silver nitrate solution was prepared by mixing 100 cc. of 0.1 N silver nitrate with 100 cc. of 0.5 N ammonium hydroxide.

b. Acetylacetone reagent was prepared as follows. Solution 1 consisted of 0.5 cc. of acetylacetone dissolved in 50 cc. of normal butanol. Solution 2 consisted of 5 cc. of 50%(w/v) aqueous potassium hydroxide in 20 cc. of ethyl alcohol. Just before using 0.5 cc. of solution 2 was added to 10 cc. of solution 1. The reagent was not stable and was made up daily from fresh acetylacetone.

c. p-Dimethylaminobenzaldehyde reagent was prepared by dissolving 1 g. of p-dimethylaminobenzaldehyde in 30 cc. of absolute alcohol and 30 cc. of concentrated hydrochloric acid. This was then diluted with 180 cc. of normal butanol.

4. Apparatus and Procedure.

Large glass cylinders, 46 cm. high and 14 cm. in diameter, were used. In the bottom of each a Petri dish was

placed. Glass supports were then inserted. On top of these a rectangular stain dish, 8.5 x 6 cm., was placed. The cylinder was sealed with a glass plate. Approximately 10 cc. of the solvent, saturated with water, were placed in the Petri dish and 20 cc. of water, saturated with solvent, in the bottom of the cylinder around the dish. Approximately 20 cc. of the solvent, saturated with water, were also placed in the upper dish. The cylinder was then sealed and allowed to come to equilibrium in the constant temperature room.

Whatman No. 1 filter paper was used. Strips, 0.5 an inch wide, and with five connected together, were carefully cut, always cutting them longitudinally in the same direction so that the fibers would always be in the same direction.

The sugar solutions were applied about 0.5 an inch below the point where the strips divided with 0.2 cc. pipettes calibrated to 0.001 cc. and the tip drawn out to a capillary tip. About 200-300 µg. of blood group material or 40 µg. of the standard monosaccharides were placed on each strip, being careful to keep the diameter of the drop about one centimeter.

The top end of the filter paper was placed in the dish under the solvent and secured with a microscope slide, see diagram, so that the sugar spots were about a fourth of an inch below the bottom of the dish. The ends of the strips

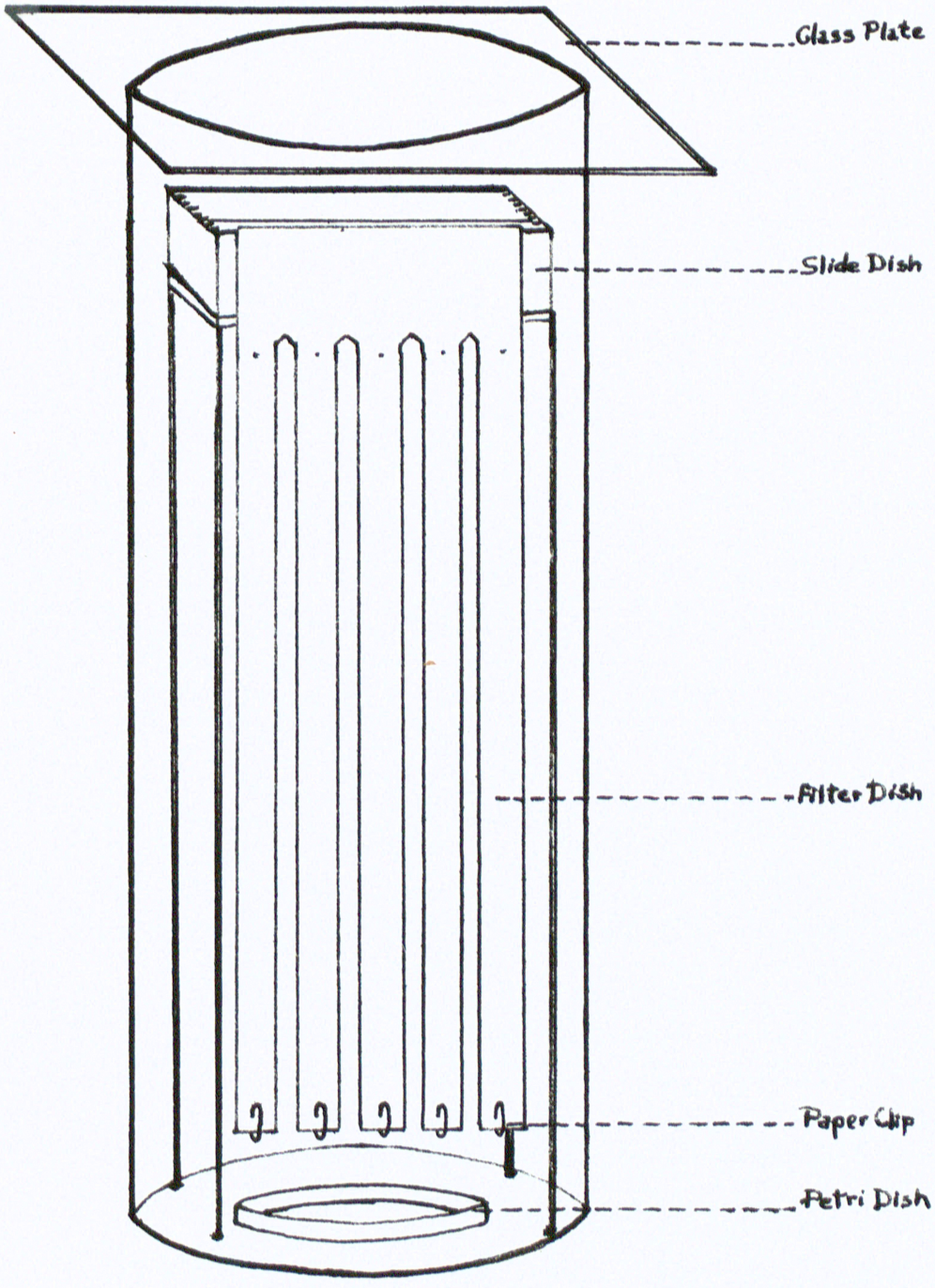


Figure 3. Apparatus for Paper Chromatography.

were weighted with paper clips so that they would not move and come in contact with the other strips or the cylinder during the process of irrigation. The cylinder was again sealed.

If it was desired to run more strips, two sets could be run simultaneously, one on each side of the dish, thus allowing ten samples to be run at the same time.

After 12-24 hours, depending upon the speed with which the solvents moved, the distance the solvents had moved was marked with a pin prick, and the strips were removed by cutting off the portion of the paper dipping in the solvent. The butyl and collidine strips were dried with an infra-red light, but this treatment affected the phenol, so the phenol strips were air dried in the dark for twenty hours. For the determination of amino sugars, all the strips were air dried in the dark.

a. Determination of Reducing Sugars. The strips were carefully and evenly sprayed with the silver nitrate mixture using an all glass spray made from heavy, capillary bore tubing.

The strips were heated in an oven at 100° C. for about five minutes. The position of the reducing sugars was then indicated by dark brown spots on a light background. The distances of the spots (and of the total distance the solvent had moved) from the point where the material was applied to

the strip were measured, and the R_f values calculated.

b. Determination of Amino Sugars. The strips were sprayed with the acetylacetone reagent and heated in an oven for five minutes at 105° C. They were then sprayed with the p-dimethylaminobenzaldehyde reagent and heated for five minutes at about 90° C. The position of free hexosamines was indicated by reddish spots on a light background. Galactose formed a brownish spot. The distances of the spots and of the total distance the solvent had moved from the point where the material was applied to the strip were measured, and the R_f values calculated.

c. Precautions.

1) The strips must hang completely free of the dish and stand or the movement of the solvent will be uneven.

2) The cylinders must be protected from mechanical vibrations and uneven temperature (such as a breeze or exposure to sunlight), and stand perfectly vertical.

3) The spray should be fine and rapidly and evenly applied.

4) Care should be taken to keep the strips from touching the others while drying, spraying, and developing.

Results:

Sample	Average R_f Values for Reducing Sugars in		
	Butyl	Collidine	Phenol
Glucose	0.19	0.50	0.41
Galactose	0.15	0.46	0.45
Mannose	0.20	0.55	0.44
N-acetyl glucosamine	----	0.60(weak)	0.36(weak)
Glucosamine·HCl	0.13	0.41	0.63
Fucose	0.29	0.52	0.66
Rhamnose	0.40	0.62	0.61
Boner Sevag	0.04	0.41	0.47
	0.13	0.50	0.66
	0.29		
Hughbanks Sevag	0.04	0.41	0.37(weak)
	0.13	0.46	0.44
	0.29	0.56	0.52
			0.64
McDowell Sevag	0.03	0.39	0.45
	0.13	0.44	0.55
	0.29	0.55	0.65
Wilder Sevag	0.04	0.43	0.46
	0.13	0.55	0.65
	0.29		
Wright Sevag	0.04	0.40	0.46
	0.14	0.44	0.55
	0.31	0.54	0.67
Calf Sevag	0.03	0.39	0.47
	0.14	0.45	0.55
	0.32	0.54	0.66

Sample	Average R_f Values for Amino Sugars in		
	Butyl	Collidine	Phenol
Galactose	0.07*	0.45*	0.45*
N-acetyl glucosamine	0.16*	0.62*	0.73*
Glucosamine·HCl	0.04	0.40	0.63
Chondrosamine·HCl	0.04	0.31	0.69
		0.37(weak)	
Boner Sevag	0.03	0.34	0.44*
	0.06*	0.41*	0.62
	0.15*	0.58*	
Hughbanks Sevag	0.04	0.35	0.45*
	0.06*	0.44*	0.63
	0.14*		
McDowell Sevag	0.04	0.35	0.43*
	0.06*	0.44*	0.63
	0.14*		
Wilder Sevag	0.04	0.34	0.45*
	0.07*	0.44*	0.64
	0.15*		
Wright Sevag	0.04	0.34	0.45*
	0.07*	0.42*	0.64
	0.17*		
Calf Sevag	0.04	0.39	0.43*
	0.06*	0.45*	0.62
	0.14*	0.61*	

* Dark color.

Discussion: The reducing sugar method seemed to be quite satisfactory, and the following conclusions could be drawn:

a. The butyl chromatogram gave three definite spots, one an unknown reducing substance with an R_f value of 0.04, one which was probably a combined spot for hexosamine and galactose, and the third which was definite for fucose. This also gave no indication for the presence of mannose which some investigators have reported in blood group substances. There was likewise no indication for glucose.

b. The collidine chromatogram also showed three spots due to hexosamine, galactose, and a third spot which could have been due to glucose, fucose, or mannose. In a few cases two of the spots overlapped giving one broad spot. On these chromatograms there was no indication of the unknown reducing substance that Partridge reported in a sample of blood group substance from hog gastric mucin.

c. The phenol chromatogram showed one spot which could have been due to either galactose or mannose, one with an R_f value of about 0.55 due to some unknown reducing substance, and a third due to hexosamine. The Hughbanks sample also gave a weak spot with a value similar to that of N-acetyl glucosamine.

By combining the findings in the different solvents, it was seen that galactose was confirmed by the phenol chromatogram when mannose was ruled out by the butyl chromatogram;

hexosamine was indicated by the phenol chromatogram; fucose was confirmed by the butyl chromatogram; and glucose was ruled out by the butyl chromatogram. No attempt was made to further characterize the unknown reducing substance. Partridge found that when the hydrolyzate of the polysaccharide was treated with 'Zeo Karb 215' the unknown material was removed, together with glucosamine. Therefore the substance was probably basic in character.

The attempt to confirm the exact amino sugar present was not as successful.

a. The R_f values in the butyl chromatogram did not correspond with those obtained by the reducing sugar method. This may have been due to the fact that the solvent mixture used had been prepared about two months previously. It did confirm the presence of galactose and N-acetyl glucosamine. The R_f values for chondrosamine and glucosamine were so similar that no further attempts were made with these solvents.

b. The collidine chromatogram gave definite indication of galactose and of N-acetyl glucosamine in two instances. However, the chondrosamine standard gave two spots, the weaker one being very similar to the spot due to glucosamine. It was not determined whether this was due to impurities in the standard or to the free base.

c. The phenol chromatogram proved to be the most satisfactory. It gave definite proof of galactose and the R_f values of the amino sugar were more similar to that of glucosamine than to that of chondrosamine.

An attempt was also made to confirm the presence of N-acetyl amino sugars since there had been several indications for their presence by the chromatography method, in addition to the fact that both amino sugars and acetyl groups had been determined chemically.

The strips were prepared in the same way, using all three solvents. When dry, they were sprayed with the p-dimethylaminobenzaldehyde reagent and dried in a 90° C. oven for five minutes. No spots could be seen on the strips at all.

The standard N-acetyl glucosamine gave a dark brown colored spot when treated with both the acetylacetone and p-dimethylaminobenzaldehyde reagents, although Partridge stated that it gave a strong purple-violet color. It likewise did not react with the p-dimethylaminobenzaldehyde reagent as expected. No explanation for this can be given.

By using two dimensional paper chromatography, Aminoff and Morgan (91) identified both glucosamine and chondrosamine but did not find N-acetyl- or N-methyl-D-glucosamine.

In conclusion, therefore, the sugar chromatography method gave definite indications for the presence of galactose, hexosamine, and fucose in the blood-group-specific materials. No gross differences between the blood-group-specific substances of different types was indicated.

More careful work with this method would probably allow complete characterization of the unknown reducing substance, hexosamines, and possibly the identification of N-acetyl amino sugars in the hydrolyzates of the blood-group-specific substances.

F. Sevag Purification of A and B Secretor Meconiums.

Seven of the A secretor meconium suspensions were combined, and five of the B secretor meconium suspensions were combined.

1. Purification. 150 cc. of each of these stock suspensions were diluted to 400 cc. with water and treated with 60 g. of sodium acetate and enough 20% acetic acid to bring the pH to 4.8. 80 cc. of carbon tetrachloride and 8 cc. of amyl alcohol were added, and the mixtures were shaken for a half hour. They were then centrifuged. The supernatants were again treated with the Sevag reagents. This was repeated another time and then twice with half the amount of the Sevag reagents.

The supernatants which then measured about 400 cc. were each treated with 2400 cc. of absolute alcohol. The precipitates were collected, washed four times with absolute alcohol, and then dissolved in 100 cc. of water.

Since the solutions still had considerable color, various attempts were made to remove the color. Charcoal was not effective. A mixture of barium chloride and sodium dihydrogen phosphate was not effective, while a mixture of barium chloride and disodium hydrogen phosphate was more so. A mixture of barium chloride and potassium phosphate was the most satisfactory, so each solution was treated with 44 cc. of 25% $BaCl_2 \cdot 2H_2O$ and 27.5 cc. of K_3PO_4 containing 23.2 g. per 100 cc. This was centrifuged and the supernatant again treated with these reagents.

The final supernatants were made up to 40% alcohol concentration to remove some of the inorganic matter, and then more alcohol was added to make the amount of alcohol six times the original volume of the supernatant.

The barium phosphate precipitates were washed with portions of water until the washes gave no further precipitation with six times their volume of absolute alcohol.

Since all of the precipitates, both from the original precipitations, A I and B I, and the washings, A II and B II, after reprecipitation and numerous washings still contained large amounts of inorganic material, each was dis-

solved in water and dialyzed against running distilled water for twenty-four hours.

The dialyzates were then treated with a few drops of sodium acetate and six volumes of alcohol. The precipitates were washed twice with 6:1 alcohol, twice with absolute alcohol, and then dried under vacuum, over P_2O_5.

2. Characterization of the Products.

Reducing sugar and nitrogen determinations were done on each sample, according to the methods as described on page 23 and page 46. Blood group activity was determined according to the methods described on page 61.

Results:

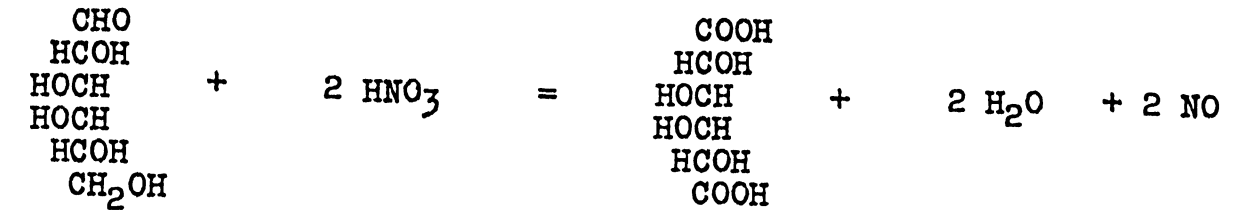
Sample	Reducing Sugar %	Nitrogen %	Ratio R.S./N	Blood Group Activity*
A I	47.6	4.92	9.66	0.4 A
A II	35.3	5.00	7.07	0.5 A
B I	46.2	4.96	9.30	53 B
B II	33.9	4.81	7.04	4.7 B

* The approximate amount of material inhibiting isoagglutination, expressed in µg. in 0.1 cc. of solution tested.

3. Enzyme Action on A II. The action of pepsin and trypsin was tested on the precipitate of the washings of the A material, fraction A II. The results are shown on page 146.

4. Mucic Acid Preparation. In order to obtain more definite proof for the presence of galactose in blood-group-

specific substances, a sample of A II was used for the preparation of crystalline mucic acid. This is the saccharic acid of the monosaccharide galactose



which differs from other saccharic acids in its solubility in water. It is only soluble one part in three hundred parts of water.

The procedure as described in Hawk, Oser, and Summerson (92) was used. 0.1 g. of blood group substance, A II, was dissolved in 2 cc. of water and treated with 2 cc. of concentrated nitric acid. This mixture was carefully heated on a boiling water bath for an hour and a half. After heating, it was immediately centrifuged to remove a little insoluble material. The clear supernatant was then allowed to stand in the ice-box for two days. A white crystalline precipitate had formed, which was separated by centrifugation and washed twice with water. After drying under vacuum over sulfuric acid, the melting point was determined.

A standard mucic acid which had been prepared from galactose by Dr. Rapoport in 1944 appeared to change at 206° C. and melted at 213-214° C. The mucic acid from the blood-group substance melted at 209° C., and a mixed melting

point was at 207.5° C.

Although this melting point was a little low, it was near enough to give definite proof that it was mucic acid, especially since the mixed melting point did not drop significantly.

5. Studies on B II. Most of the precipitate from the washings of the B material, B II, was sent to Dr. Jesse W. Huff, the Director of Biochemical Research at Sharpe and Dohme, for an anaphylactogenic test. He found the B potency of this material to be 1:729, and that it did not contain A substance activity.

The results of the anaphylactogenic test are shown on the next page. He concluded as follows: "Five of the sensitized animals in the anaphylactogenic test were challenged with normal human plasma (N.H.Pl. in the table) instead of the B substance, and the severe reaction obtained would perhaps indicate that this material is not entirely free of protein."

Discussion: This method of purification gave a low yield but the products and their solutions were much less colored and the reducing sugar to nitrogen ratio was higher than that of any other preparation.

Project 960 July 10, 1950

Human Blood Group B Substance (Rapoport) Anaphylaxis

Guinea pigs sensitized with 1 ml. of 1% solution I. P. Challenged 3 weeks later I.V. with 1% solution of B substance and with normal human plasma.

G.P. #	Challenged with	Dose	Reaction
1		3	4+
2		3	4+
3		2	3, 4+
4	B Substance	2	2, 3, 4+
5		2	3, 4+
6		1	2, 3, 4+
7		1	2, 3, 4+
8		1	2, 3, 4+
9		1	2, 3, 4+
10		0.5	1, 2, 3 (5'), 4+ (8')
Control	(not sensitized)	3	No reaction
Control	(not sensitized)	0.5	No reaction
1		3	2, 3, 4+
2		3	1, 3, 4+
3	N.H.Pl.	2	2, 3, 4+
4		1	1, 2+ (survived)
5		0.5	1+ (survived)
Control		3	No anaphylactic reaction

4+ = death. All animals except where indicated died in 5' or less.

G. Conclusion.

1. The blood, saliva, and meconium of seventy babies was collected. The meconium from all babies with blood types A, B, and AB contained the corresponding blood-group-specific substances if the baby was a secretor of blood-group-specific substances as shown by its saliva.

In testing twenty-one babies of type O, eleven were found to be secretors, eight non-secretors, and two possessed blood group activity in the meconium, although no sign of activity was found in the saliva. In all cases the O activity was weaker than the A and B activities, which was not surprising because of the weak antigen activity of the O substance.

2. When meconium was tested with serum containing three to four agglutinating units of anti-Rh_o (anti-D), there was no definite indication of Rh activity, but when more dilute serum containing only one or two agglutinating doses of anti-Rh_o agglutinin was used, Rh activity was definitely observed. This was extremely interesting since Rh activity was found in amniotic fluid by Witebsky using similar techniques. Since one Rh_o^- baby possessed Rh_o^+ activity in its meconium, this subject requires further study.

3. No indication of the presence of M and N activity in meconium was seen, although it is possible that, as with Rh, the methods that were used were not sufficiently sensitive

to detect the activity.

4. The amniotic fluid and meconium were found to contain the blood-group-specific substances corresponding to the blood type of the baby and not corresponding to the blood type of the mother. The blood group substance of meconium and amniotic fluid is, therefore, ultimately of fetal origin, but whether this is secreted by the amnion or excreted in the fetal urine into the amniotic fluid and the amniotic fluid swallowed and concentrated to form meconium is not known.

5. The following table summarizes the chemical data on the crude meconium samples and the Sevag purifications:

Sample	Reducing Sugar %	Amino Sugar %	Methyl Pentose %	Nitrogen %	Ratio R.S./N
Crude Meconiums					
Human	25.6-31.5	14.1-19.7	3.0-5.9	5.9-7.3	3.85-4.92
Calf	12.0	6.8	1.0	6.2	1.93
Dog	5.6	4.1	1.4	9.7	0.58
Sevag Purifications					
Human	32.0-41.6	19.4-24.8	5.2-8.4	5.9-7.2	5.07-6.71
Calf	32.0-36.6	22.4-22.8	4.6	5.4-6.4	5.0 -6.78
Dog	14.1	9.8	---	7.8	1.81

All of the normal human meconium samples analyzed appeared to be very similar chemically. The calf meconium and dog meconium, although possessing the same constituents had considerably less carbohydrate, possibly because they were removed from the intact intestine rather than being

excreted normally, thus preventing as long a contact with the intestinal enzymes.

Sevag purifications in every case greatly increased the carbohydrate content, probably by removing the fat and traces of proteins and other impurities with high nitrogen contents.

The dog meconium still contained less carbohydrate. This probably was due to the fact that it was removed from the entire intestine and as shown later, meconium in the upper part of the intestinal tract contained large amounts amounts of nitrogen, probably in the form of protein.

The methyl pentose to reducing sugar ratio gave indications that it might be characteristic of the blood type. Materials with A or B activity had higher ratios than the type O materials and substances obtained from non-secretors. It is possible that the amount of methyl pentose present governs the type of activity which will be exhibited.

6. Paper chromatography of the sugars gave indications for the presence of galactose, hexosamine, and fucose. No gross differences between the blood-group-specific substances of different types were observed.

More careful work, probably using two-dimensional chromatograms, will be necessary to identify the hexosamine components and to determine if N-acetyl sugars were present.

7. The presence of galactose was confirmed by the preparation and isolation of mucic acid.

8. Sevag purification of type A and B secretor meconiums followed by treatment with $BaCl_2$ and K_3PO_4 yielded products which had higher reducing sugar to nitrogen ratios and per cent of reducing sugar than any other purification.

Sample	Reducing Sugar	Nitrogen	Ratio R.S./N	Blood Group A Activity*
Morgan (p.160)	57	5.72	9.96	0.2
A I (p.200)	47.6	4.92	9.66	0.4
A II (p.200)	35.3	5.00	7.07	0.5

* The approximate amount of material inhibiting isoagglutination, expressed in μg. in 0.1 cc. of solution tested.

Sharpe and Dohme studied the second fraction of the B substance and found that it produced fatal anaphylactic shock in guinea pigs, sensitized with the substance. Undoubtedly the first fraction would have been less antigenic.

VI. Comparison of Clinical Samples of Mucus Secretions.

A. Aim of the Investigation.

The work on the comparison of normal meconium and meconium ileus showed a very large and characteristic difference in the carbohydrate-nitrogen ratios on the crude products, a difference which became even more pronounced after various methods of purification and fractionation had been employed. Because of these large and characteristic differences, the carbohydrate-nitrogen ratios in other pathological meconiums and the mucus secretions from other regions of the body were determined.

Of particular interest were the samples of mucus secretions from patients suffering from cystic fibrosis, of which meconium ileus is the first and most severe form. These patients have a great deal of mucus in their lungs which often causes respiratory disturbances and frequently, upon autopsy, their gall bladders are found to contain a characteristic 'white bile', which is a white, almost clear mucus. It is most likely formed by the mucus glands in the gall bladder, which normally contribute to the formation of the bile secretion.

Upon the advice of Dr. Rapoport, Dr. Roxie T. Mudgett of the Pathology Department of the Cincinnati General Hospital devised a simple method which gave indications of

being useful as a simple diagnostic tool, definitely in cases of intestinal obstruction in newborns due to malformations of the intestinal tract or to meconium ileus, and possibly in patients suffering from cystic fibrosis of the pancreas. It was based upon the solubility of these secretions in 50% alcohol.

It was also believed that by comparing the meconium at different levels in the intestines, the mucus that infants frequently cough up, and the amniotic fluid, further information concerning the formation of meconium might also be obtained.

B. Characterization of the Secretions.

Reducing sugar was determined according to the method described on page 23, nitrogen according to the method described on page 46, and blood group activity as described on page 61.

The method which Dr. Mudgett found to be the most satisfactory was to make smears of the samples to be tested on microscope slides and allow them to dry. One end of the smear was then immersed in 50% alcohol overnight. Upon removal, the slides could be dipped in crystal violet stain, although it merely served to emphasize the difference between the dissolved and the undissolved portions on the

slide. In the table, - means that all of the material which was immersed in alcohol was dissolved; ± means that some of the material dissolved but a considerable amount did not; and + means that no appreciable portion of the smear was soluble in 50% alcohol.

Sample	CHO/N Ratio	Smear Test
1. Staggs, mucus from the gall bladder	9.0	
2. Ledman, mucus from the gall bladder	8.5	±*
3. West, mucus from the gall bladder	5.71	+
4. Worley, meconium plug	4.84	-
5. Oliver, vaginal secretion	3.58	+
6. Young, meconium plug	3.1	-
7. Wilder, normal meconium	4.68	±
8. Hughbanks, normal meconium	4.61	±
9. Boner, normal meconium	3.85	±
10. McDowell, normal meconium	4.26	-
11. Thompson, normal meconium	4.63	-
12. Allen, normal meconium	4.18	-
13. Fetter, normal meconium	3.88	-
14. Kellum, normal meconium	4.65	-
15. Wright, normal meconium	4.49	±
16. Spradlin, normal meconium	4.53	-
17. Williams, normal meconium	4.91	-

* Dr. Mudgett got a negative test on this sample.

Sample	CHO/N Ratio	Smear Test
18. Weigel, normal meconium	4.92	-
19. Fowler, first meconium stool	2.1	+
second meconium stool	1.15	+
20. Taylor, meconium plug	1.3	+
21. Chance, kidney cyst	1.4	+
22. Premature, meconium	1.4	+
23. Knight, white meconium	0.88	+
greenish meconium	1.68	+
24. Stamper, normal (?) bronchial secretions	1.05	+
25. West, bronchial secretions	1.00	+
26. Cain, bronchial secretions	0.74	+
27. Dawson, bronchial secretions	0.43	±
tracheal secretion I	0.61	+
tracheal secretion II	0.41	±
28. Davidson, meconium ileus	0.67	
29. Zinser, meconium ileus	0.60	+
30. Hill, bronchial secretions	< 0.20	+

Discussion:

1. This was a sample of mucus material which was removed from the gall bladder of Gail Staggs on autopsy. She had cystic fibrosis of the pancreas. Evidently this material was a very pure muco-polysaccharide. It was a colorless mucus mass. The high carbohydrate to nitrogen ratio does not have a

ready explanation.

2. Lois Ledman was a nine-year-old girl who had fibrocystic disease of the pancreas, cirrhosis of the liver, and bronchiectasis. The sample of 'white bile' was a colorless mucus secretion removed from the gall bladder.

3. Bruce West was a nine-month-old baby with fibrocystic disease of the pancreas and involvement of the lungs which consisted of peribronchial infiltration and generalized emphysema. The gall bladder sample was the white mucus, characteristic of these patients. The bronchial material was a thick mucus, contaminated with blood.

4. This was a meconium plug from Louise Worley, a normal infant. It had the light color characteristic of true meconium plugs.

5. This was a colorless mucus secretion from the vagina of Ora Lee Oliver, a normal infant. This is frequently found in newborn infants and is caused by the maternal hormones.

6. This was a light-colored meconium plug from Opal Young, a normal infant.

7 - 18. These were all samples of normal meconium.

19. Michael Fowler was a seven months premature infant with

a stenosis of the middle portion of the jejunum. The two meconium stools were in the form of fairly hard lumps about the size of small peas. The second stool had a slight green color. Unlike the usual mucous secretions these samples remained almost insoluble in water. The supernatants became turbid when 20% trichloroacetic acid was added, and a little cloudy when a drop of glacial acetic acid was added. Heat did not appear to cause further coagulation. After standing for several days with trypsin and pancreatin in a sodium bicarbonate solution at room temperature, some of the material became soluble, but never completely. The material did not dissolve in base. Autopsy revealed a stenosis of the entire small intestine which was small like a cat intestine. Some of the meconium contained a little bile, so the high nitrogen values were probably due to partial obstruction and the premature birth of the child. There does not seem to be any reasonable explanation for the unusual characteristics and behavior of the meconium.

20. Jackie Taylor was an eight month premature, who had a very thick tenacious meconium plug. There was an X-ray suggestion of cystic fibrosis but tryptic activity was demonstrated. A suspension of some of the plug gave a precipitate with 20% trichloroacetic acid, and when a drop of glacial acetic acid was added a little heat coagulable

material could be demonstrated. This would indicate that more protein was present than normal, but it might be accounted for by the premature birth of the child.

21. Infant Chance had polycystic kidneys, an imperforate anus, double vagina and uterus, and a common cloaca. In an exploratory operation a cyst which was posterior to the bladder was drained. The material was a thick cloudy mucoid. Because of the extensive malformations, no explanation of the high nitrogen content could be made.

22. This was an apparently normal premature from about a six month pregnancy. It weighed about 1000 g. on birth. It lived four days, but did not have a meconium stool until the fourth day when it had a small one. The meconium tested was removed on autopsy from the entire intestine.

23. Roger Lee Knight was a seven month premature whose pathological diagnosis on autopsy was atresia of the duodenum, collapse of the terminal duodenum, small and large bowel, slight dilatation of the extrahepatic bile ducts and gall bladder, slight fibrosis of the pancreas, and peritoneal adhesions from the transverse colon to the duodenum and gall bladder and from the duodenum to the retroperitoneum. The first meconium which was passed about eight hours after birth was white and 'millet seed size.' On autopsy the

obstruction of the duodenum was seen to be distal to the pancreatic duct, but a little of the meconium was slightly green, so a small duct or branch of the bile duct may have been opened below the obstruction. The high nitrogen content may be accounted for by the premature birth of the child, the slight fibrosis of the pancreas, and the obstruction of the bowel preventing the contact of the proteolytic enzymes with the meconium. The higher ratio in the meconium with a greenish color would indicate that some enzyme action had probably taken place.

Suspensions of both the white and green meconium gave a definite precipitate when treated with a drop of glacial acetic acid and heated. This indicated the presence of heat-coagulable protein similar to that found in meconium ileus.

24. This was a sample of bronchial secretions received from Gloria Stamper on bronchoscopy of a piece of foreign material.

25. See 3.

26. This was a sample of bronchial secretions from Alma Cain, an eight-year-old girl who probably had bronchiectasis, and was a possible fibrocystotic, although a definite diagnosis was not made. This might explain the high nitrogen content of the bronchial secretions.

27. Terry Lee Dawson was a two and a half-year-old boy who had pancreatic cystic fibrosis. Thick ropy tenacious secretions which were impossible for the child to cough up were shown on bronchoscopy. Tracheotomy had to be performed later due to laryngeal edema. All of the secretions had a ratio which seemed characteristic of cystic fibrosis.

28. Infant Davidson had meconium ileus. The sample we received had been washed out with saline.

29. This sample of meconium ileus was received from Infant Zinser on autopsy.

30. Daryl Hill was a patient with croupe and bronchopneumonia. He also had some asthmatic condition.

C. Partial Characterization of Amniotic Fluid.

Three samples of amniotic fluid were studied chemically in order to determine if it could possibly act as one of the sources of meconium through fetal swallowing.

The reducing sugar, total nitrogen, and non-protein nitrogen methods described on pages 23, 46 and 48 were used.

Results:

Sample	Total R.S. *	Free R.S. *	Non-prot. R.S. *	Total N *	N.P.N. *	Free R.S.	Non-prot. R.S. %	N.P.N. %
Berry**	305	---	154	136	91	---	50.5	66.9
George**	250	107	108	265	145	42.8	43.2	54.7
Caudill	300	129	162	303	148	43	54	48.8

* µg. per 0.5 cc. sample of the original fluid.
** Artifically collected fluid from unruptured membranes.

Sample	Ratio Total R.S./ Total N	Ratio Protein R.S./ Protein N	Blood Group Activity*	Smear Test
Berry	2.24	3.36	5 A	
George	0.94	1.18	---	±
Caudill	0.99	0.89	3 A, 3 B	±

* The approximate amount of protein reducing sugar, expressed in µg., present in the 0.1 cc. of solution tested, inhibiting isoagglutination.

Discussion: Since the free reducing sugar was approximately equal to the non-protein reducing sugar, it was possible that the blood group activity might be associated with the protein

fraction. Making this assumption it was seen that the blood group activity, calculated on the basis of reducing sugar present in the solution inhibiting isoagglutination, was similar to that found in meconium.

D. Partial Characterization of Mucus Coughed up by Newborn Infants.

Three samples of the mucus coughed up by newborn infants were analyzed for their nitrogen and reducing sugar contents according to the methods described on page 46 and page 23, to see if these materials might feasibly be one of the sources of meconium.

Results:

Sample	CHO/N Ratio	Smear Test
Sadler, clear mucus	2.19	+
Crowder, bloody mucus	1.77	---
Watson, bloody mucus	0.69	---

Discussion: The Watson and Crowder infants were apparently normal full term babies, while the Sadler infant was a premature, weighing about four pounds.

E. Partial Characterization of Meconium at Different Levels of the Intestinal Tract

Infant girl Coe was born about two months prematurely and weighed 2 pounds 2 ounces. It was autopsied about five hours after its death which was due to its premature birth

and fetal atelectasis. The intestine was divided into sections and the meconium expressed. Each sample was then analyzed for its reducing sugar and nitrogen content and its solubility in alcohol according to the slide test.

Results:

Sample	Ratio CHO/N	Smear Test
I. Rectum, dark-green, viscid, typical meconium	6.08	-
II. Sigmoid colon, 1 inch, typical meconium	5.12	-
III. Sigmoid colon, 1 inch, typical meconium	6.16	-
IV. Descending colon, 2 inches, typical meconium	5.59	-
V. Descending colon, 2 inches, lighter, less viscid	3.92	-
VI. Transverse colon, 2 inches, less viscid, lighter	4.43	+
VII. Transverse and ascending colon, 5 inches, light, yellowish, almost fibrous	2.53	+
--- Appendix		
VIII. Ileum, 5 inches, light, slightly green, less gummy	1.48	+
IX. Ileum, 8 inches, light, no green	1.57	+
X. Jejunum, 9 inches, more like mucus	1.14	+
XI. Jejunum, 9 inches, more like mucus	1.10	+
XII. Duodenum, 18 inches, light, fibrous	0.75	+

Discussion: The composition of meconium changes as it progresses down the intestinal tract, probably due to the action of the proteolytic enzymes on the protein or amino acid portion.

F. Partial Characterization of the Transitional Meconium.

Four samples of meconium passed 48-72 hours after birth by apparently normal infants were collected. All of the samples were smelly. They were each analyzed for reducing sugar and nitrogen content and their solubility in 50% alcohol according to the slide test.

Results:

Sample	Ratio CHO/N	Smear Test
1. Upshaw	5.02	±
2. Ragland	4.85	±
3. McDonough	4.69	-
4. Wiggins	6.26	-

Discussion: These samples indicated that the composition of meconium continued to change, by the nitrogen value continuing to drop, and the carbohydrate ratio continuing to rise.

G. Conclusion.

1. Mucus from the gall bladder of fibrocystic patients, 'white bile', had a very high carbohydrate to nitrogen ratio, but in some cases gave a positive reaction with the smear test. It is possible that although the ratio is high, the normal ratio is still higher. This could not be checked since no normal samples were available.

2. The smear test appeared to be a reliable test for abnormal types of meconium and to coincide with the carbohydrate to nitrogen ratio. Normal meconiums had a ratio of 3.85-4.92 and gave - or ± smear tests, as did meconium plugs from otherwise normal babies. However, the meconium from a premature, two patients with intestinal stenosis, a plug from a baby that possibly had cystic fibrosis, and two patients with meconium ileus had carbohydrate to nitrogen ratios below 2.1 and gave + tests with the smear test.

3. Since cystic fibrosis may be the result of a pathological condition of all the mucous membranes, samples of the bronchial and tracheal secretions of several fibrocystic patients were studied to see if they behaved like meconium. All had a very low carbohydrate to nitrogen ratio and gave + or ± smear tests. However, one bronchial secretion obtained from a child which had aspirated a foreign body and another from a child with croupe and bronchopneumonia

also had low ratios and gave positive smears. Therefore, no conclusions can be drawn from these results until more normal bronchial secretions can be studied. It is possible that any infection or irritation increases the nitrogen content and will invalidate this test.

4. It was found that amniotic fluid had blood group activity and contained a considerable amount of protein, which apparently was bound with a carbohydrate, suggesting that the blood group activity here might be associated with a type of mucoprotein. Although this was not confirmed, it appeared plausible since most of the non-protein reducing sugar was accounted for by free reducing sugar. Kabat and his co-workers (49) did a peptic digestion of amniotic fluid to obtain the blood group material.

5. The mucus coughed up by newborn infants had a fairly low carbohydrate to nitrogen ratio.

6. The carbohydrate to nitrogen ratio was found to rise from less than one in the duodenum to 6.08 in the rectum in samples of meconium removed from various levels of the intestines of a premature infant on autopsy.

7. Several samples of transitional meconium stools had carbohydrate to nitrogen ratios of 4.69-6.26 in comparison with the range of 3.85-4.92 in normal meconium, suggesting that they were further acted upon by enzymes in the intes-

tinal tract or produced by bacteria.

8. From these ratios in amniotic fluid, coughed-up mucus, the levels of meconium, and the transitional stools, it would appear that the formation of meconium could be, at least in part, due to the accumulation of swallowed amniotic fluid. Probably this is not the only source of the meconium, but at least from these data could contribute to its formation. This would confirm the theory that meconium is the result of the accumulation of swallowed amniotic fluid, secretions of the gastro-intestinal tract, bile, and enzymes.

VII. Summary

A. Normal meconium and meconium ileus were compared chemically. Normal meconium was found to contain little protein and large amounts of carbohydrates, probably in the form of a polysaccharide-amino acid complex. Meconium ileus consisted largely of protein that appeared bound with small amounts of carbohydrate, probably in the form of a muco-protein.

B. The polysaccharide in normal meconium was found to contain galactose, hexosamine, and methyl pentose. The largest amount of normal meconium consisted of material which was capable of inhibiting isoagglutination. No method was found in which the carbohydrate and nitrogen components could be separated without destroying this activity, although it was possible to fractionate meconium into several fractions which had varying ratios of reducing sugar to nitrogen and amounts of blood group activity.

C. In contrast, meconium ileus consisted mainly of a protein which was firmly connected to a polysaccharide. It did possess blood group activity so it must have the same essential groupings exposed as blood group substances from other sources.

D. Normal meconium and meconium ileus were further compared by studying their behaviour in dialysis, ammonium sulfate and alcohol fractionations, and peptic and tryptic digestions.

E. The purest fractions of the Sevag and phenol purifications of normal meconium, when compared with the data on the homogeneous blood-group-specific substances prepared from pseudomucinous ovarian cysts by Morgan (87), apparently still contained impurities, some of which contained nitrogen, if substances from different human sources can be compared. The methyl pentose content was much lower than that found in substances from other human sources.

F. The blood-group-specific substance found in meconium corresponded in all examples studied with the blood type of the baby and the baby's ability to secrete blood group substance in its saliva, except two cases of type O babies who had O substance in the meconiums but no trace of O activity in their saliva.

G. Evidence for the presence of Rh_o^+ material in meconium was obtained, but no indication of M or N activity was found; however, the methods used may not have been sensitive enough.

H. The amniotic fluid and meconium were found to contain the blood-group-specific substances corresponding to the blood types of the baby and not that of the mother.

I. Samples of human, calf, and dog meconiums were studied and compared chemically before and after Sevag purification. The methyl pentose to reducing sugar ratio of human meconiums with type A or B activity was higher than the ratio in meconiums obtained from type O individuals or non-secretors.

J. Paper chromatography indicated the presence of galactose, hexosamine, and fucose. No gross differences were observed between materials with different types of activity.

K. The presence of galactose was confirmed by the preparation and isolation of mucic acid.

L. A smear test depending upon the solubility of the substance in 50% alcohol appeared capable of detecting abnormal types of meconium. These results coincided with the low carbohydrate to nitrogen ratios of the abnormal meconiums. Preliminary results indicated that it may be useful as a diagnostic method of detecting cystic fibrosis.

M. The carbohydrate to nitrogen ratio was found to rise from less than one in the duodenum to 6.08 in the rectum in samples of meconium removed from various levels of the intestines of a premature infant on autopsy.

N. From the carbohydrate to nitrogen ratios in amniotic fluid, coughed-up mucus, the levels of meconium, and the transitional stools, it appeared that the formation of meconium could be, at least in part, due to the accumulation of swallowed amniotic fluid, lending support to the theory that meconium is the result of the accumulation of swallowed amniotic fluid, secretions of the gastro-intestinal tract, bile, and enzymes.

VIII. Suggestions for Future Work

1. Study of the bile pigments in meconium.

2. Study of the effect of enzymes other than pepsin and trypsin on the digestion of normal meconium and meconium ileus, since it apparently was not the lack of either of these enzymes which was responsible for the altered composition of meconium ileus.

3. Alcohol fractionation of the Sevag and phenol purified products to obtain more homogeneous products and to see if these purification procedures removed all but one of the fractions.

4. More careful comparison of the various meconium purifications with Morgan's homogeneous product from human pseudomucinous ovarian cysts to see if these materials from different sources were the same

5. Characterization of the various purifications for homogeneity by solubility, ultracentrifuge, and electrophoretic methods.

6. More complete chemical studies: (a) check the methyl pentose method more carefully to see if meconium does have a lower methyl pentose content than the blood group substances

from other human sources, (b) study of the hexosamine component to see if one or both glucosamine and chondrosamine were present, (c) study to determine if N-acetyl-hexosamine were present, (d) determination of the amino acids, (e) determination of optical rotation, (f) structural studies using methylation and similar techniques, (g) study of the action of blood group enzymes on the material to see what components were removed when the activity was destroyed, and (h) study of the breakdown of the blood group substances by the action of alkali and other methods.

7. Comparison of the fatty material in meconium with the vernix caseosa to see if this would give an indication to the formation of meconium.

8. Chemical studies on the amniotic fluid, the mucus coughed up by newborn infants, and the meconium levels to see if further information on the formation of meconium could be obtained.

9. Study of the abnormal meconiums in order to find out what caused the differences in their composition.

10. Study of the bronchial and other abnormal mucus secretions of patients with cystic fibrosis to see (a) if the smear test could be used diagnostically, and (b) if the cause of cystic fibrosis could be determined.

11. More sensitive tests for the presence of the M and N agglutinogens.

12. Further study of the presence of Rh_0^+ agglutinogen in meconium, (a) to see if its occurrence could be correlated with the blood type of the infant or mother, (b) especially in infants with erythroblastosis, to see if any information as to its cause could be obtained, (c) to see if it could be separated from the blood-group-specific materials, A, B, and O, (d) chemical study of the factor, and (e) uses for the material such as neutralization of antibodies.

13. Anaphylactic tests with the blood group materials to see if they can be freed of anaphylactogenic material sufficiently to be used in the preparation of blood for transfusion purposes.

14. Experiments with the amniotic fluid to see if it were swallowed, such as injecting a dye or carbon into the amniotic fluid in rats and seeing if these substances were present in the meconium.

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