# A CHROMATOGRAPHIC STUDY OF BILIRUBIN

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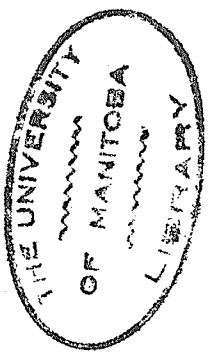

## ACKNOWLEDGMENTS

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## ABSTRACT

The bilirubin pigments found in human blood serum have been studied by means of the reverse phase chromatographic technique. The technique itself was found to be effective only in the qualitative separation of the pigments. From the evidence of this study, it can be stated that there are three distinct bilirubin pigments, only one of which can be properly called bilirubin, the other two being of a "direct" acting nature as judged by their coupling reaction with diazo reagent. Evidence is given in support of the theory that direct acting bilirubin is attached to protein in vivo, and that this complex increases the ability of the pigment to resist oxidation by exposure to air. It has been demonstrated that greatly increased levels of bilirubin in the body are not alone responsible for the development of kernicterus in the newborn rat, and that other unknown factors are the toxic agents in this condition.

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# PART I

## INTRODUCTION

Since the year 1916, when Hijman van den Bergh and Muller (1916) discovered that the diazotization reaction could be used to indicate the presence of two different states of bilirubin in blood serum, there has been a constant search to discover the nature of these two or more fractions. The development of any new technique gives further impetus to this search, and accordingly Cole and Lathe (1953) adapted the chromatographic technique introduced by Howard and Martin (1950) in an attempt to separate serum bilirubin into its component fractions.

The solubility of bilirubin in serum at pH 7.35 has posed a major problem, since in vitro pure bilirubin is only soluble at pH 8.0 or higher. The nature of the diazo reaction in water and in alcohol, and the ability of bilirubin to remain in solution at physiological pH values seem to be closely connected. The present study was carried out with a view to discovering if the chromatographic technique was useful for separating bilirubin fractions and if it could be used to elucidate the vexed question as to how bilirubin is carried in the blood stream.

## REVIEW OF THE LITERATURE

The determination of the structure of the bilirubin

molecule has been established within the last twenty years, due chiefly to the work of Fischer (1937) and of Lemberg (1949). Each has approached the problem from a different angle. Lemberg was able to synthesize bilirubin from haemin in 1935 and Fischer synthesized both bilirubin and biliverdin from simple pyrrole compounds in 1942.

The accepted formula for bilirubin as synthesized by Fischer is a tetra-pyrrole di-carboxylic acid having an open chain structure. The degradation of bilirubin by resorcinol fusion by Fischer (1931) has led to a further understanding of the mechanism by which the molecule is able to enter into coupling reactions with diazonium chloride.

This coupling reaction is of particular importance in biological chemistry because it has been used to measure the quantity of bilirubin occurring in blood. The bilirubin is capable of being split at the central carbon atom and because of its asymmetrical nature, the two resulting dipyrroles will be different. Thus one half of the tetrapyrrole forms a dipyrrole having a free alpha position, while the other half has no such free position, it being filled by a methoxy group. Because of the asymmetry of the bilirubin molecule, the molecule can split at either side of the central $CH_2$ group, and therefore for each of the two active dipyrroles formed, there will be an inactive form. The two possible active forms are shown in Fig. 1. These are neoxanthobilirubinic acid and isoneoxanthobilirubinic acid. The phenyl diazonium chloride can couple at either of two

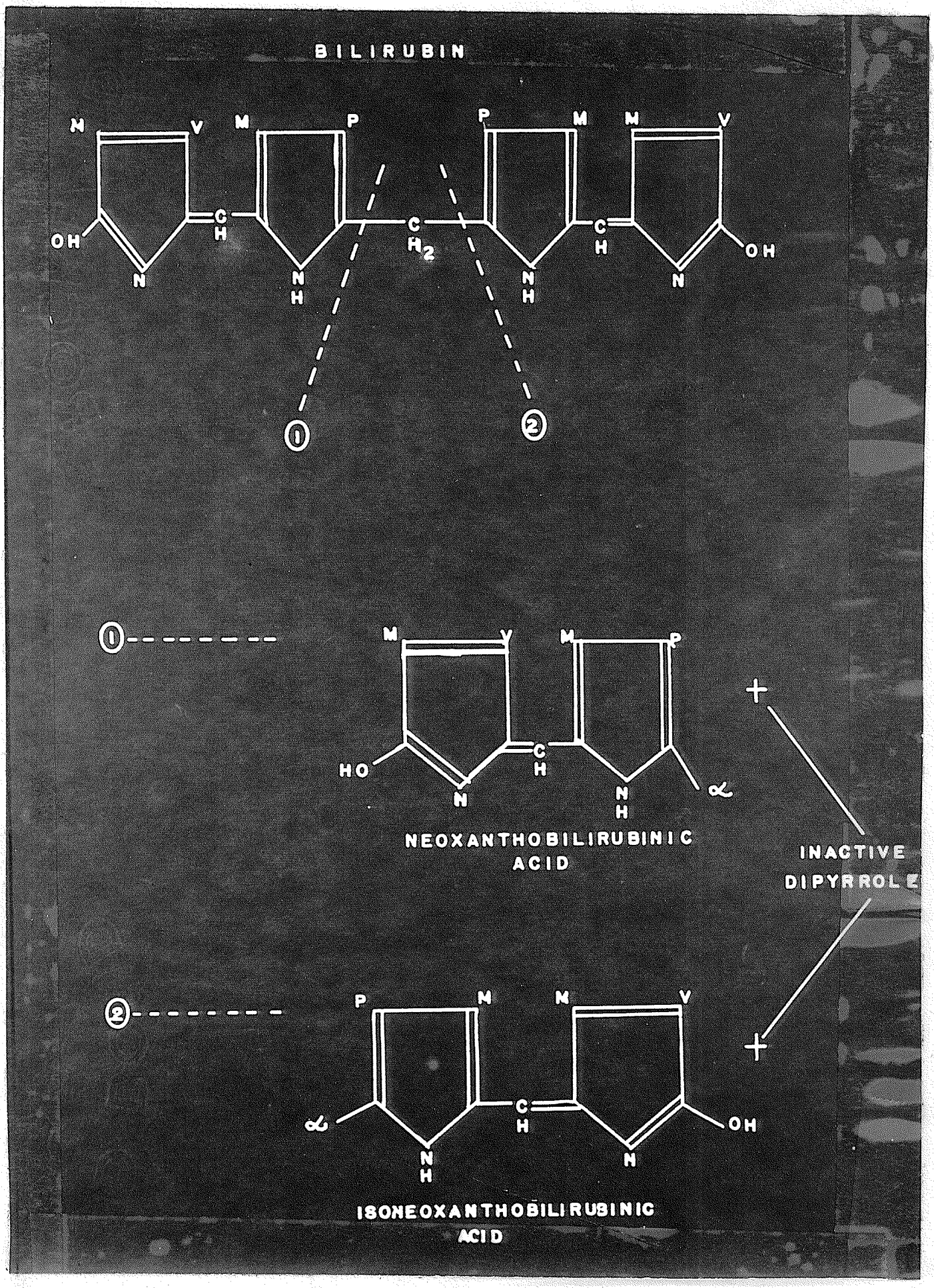


Figure 1

**The Chemical Structure of Bilirubin**

free alpha positions. The resulting azo pigment which is used for measurement is thus a mixture of two azo pigments which differ only in the positions of the methyl and vinyl side chains. These mixed azo pigments have not thus far been separated.

The action of the diazotized sulphanilic acid is to split the tetrapyrrolic molecule and release free alpha positions and then to couple to form the diazonium salt. Van den Bergh in 1916 utilized this chemical reaction to demonstrate the quantity of bilirubin in blood serum. He found, however, that in most cases simple treatment of the serum with the diazotized acid did not produce the azo pigment, but that it was necessary to have at least 50% of alcohol present in the reaction mixture before coupling took place. By accident he discovered that the serum of certain of his patients had a type of bilirubin which was capable of undergoing diazo coupling without the presence of alcohol. He called this type of reaction without alcohol, the "direct" reaction and that requiring alcohol the "indirect" reaction.

Since then there has been a good deal of speculation about the meaning of these two types of reaction and many theories have been evolved to explain why one blood serum can readily react while another serum requires alcohol or at least alcoholic groups.

The theories fall generally into three groups. First are the theories that claim that bilirubin is actually of

two different types. Secondly, the theory that in some cases catalysts are present in the blood which allow bilirubin to couple directly with diazo reagent without the presence of alcohol. Thirdly, that indirect bilirubin is attached to blood proteins or lipids in such a way as to prevent the coupling with diazo reagent, whereas direct bilirubin has no such attachment. In the former case it is necessary to break this attachment by using alcohol first before the reaction can take place.

In reviewing the literature it seems reasonable to depart from a chronological order in favor of a division along the lines of the above mentioned three principal theories.

The first theory--that bilirubin is really of two quite different types--is supported by Najjar (1952) who has been able to crystallize two different forms of bilirubin from serum. The crystals of bilirubin obtained from the serum of cases of haemolytic jaundice are well formed, fine long needles, while those obtained from cases of obstructive jaundice are thick, short and rod shaped. Najjar believes that whatever the differences in bilirubin, they are unrelated to the nature of the protein which is combined with the pigment. He claims it is possible to change one form into the other by manipulation of the pH and the temperature. He suggests that direct acting bilirubin is a bilirubin-metal--protein ternary complex and that the function of alcohol in

the diazo reaction is to catalyze the formation of this complex.

A recent report by Childs (1956) lends weight to the bilirubin-metal complex theory. Childs claims to have been able to convert direct acting bilirubin to the indirect type by the addition of a metal binding agent, Versene (etylene diamine tetra acetic acid). Since the only known effect of adding Versene is in its metal binding ability, it is thought to be an indication of the presence of bilirubin-metal in serum.

Yamaoka and Kosaka (1953) in their study on the nature of direct and indirect bilirubin conclude that the indirect form is a dibasic acid with free acid radicals on the propionic acid side chains while the direct is the salt or ester of the dibasic acid.

Other explanations have been advanced to account for the two different reactions of serum. Kuster (1909) believes that bilirubin in serum is present as a keto-enol system, the keto type being the more stable, slow reacting type found in haemolytic jaundice. The enol type is found in cases of obstructive jaundice. The shift from keto to enol form can be accomplished by dissolving the keto form in dilute alkali and then salting out with concentrated alkali. Collinson and Fowweather (1926) consider the direct bilirubin to be an alkali salt, probably the ammonium salt, but perhaps the sodium salt. They claim to have been able to convert an indirect bilirubin to a direct form, by dissolving bilirubin

in dilute alkali and precipitating direct bilirubin out by the addition of a concentrated solution of the same alkali. Other workers have not been able to confirm this work.

Gardikas (1947) believes that the fact that both forms of bilirubin are attached to protein, eliminates the theory that the difference between direct and indirect is attachment or non-attachment to protein. He showed that digestion of serum with pancreatin under optimal conditions does not alter the indirect or direct diazo reaction. He suggests that indirect bilirubin may be a precursor of direct bilirubin in its formation from haemoglobin. The indirect may still be attached to the globin part of the molecule.

Hunter (1930) summarized the differences in properties of the two types of bilirubin. He concluded that bilirubin, being a dibasic acid, can occur as such or as a mono or dibasic salt. At the pH of blood it is most probably in the form of the mono basic salt, sodium hydrogen bilirubinate. In this respect he is in agreement with the hypothesis of Collinson and Foweather.

The second theory on the nature of the bilirubin diazo reactions, is that a catalyst is present in the blood which is capable of slowing down or of accelerating the diazotization of serum. Gray (1947) suggests that in regurgitation jaundice there may be a secretion into the blood stream of some unknown catalyzing factor. The catalyzing factor could change the nature of the serum by making it

more hydrophilic, and also alter the effect of the diazo reagent on the serum. No such catalyst has so far been found. Cantarow (1944) also believes that a catalyst is present in serum of patients having obstructive jaundice. He has mixed sera from obstructive and from haemolytic jaundice in various proportions and has found that the values for direct bilirubin are enhanced beyond the sum of the two components. Klatskin and Drill (1950) suggest that the rate of diazo coupling is determined by the concentration of bilirubin in the blood and by unknown chemical and physical factors in serum and not because there are variable mixtures of two types of bilirubin having different reaction rates. They have confirmed Hunter's work that bilirubin in chloroform does not react with the diazo reagent while the addition of an extremely small percentage of alcohol causes the reaction to take place. Evidently the composition of the solvent is of importance, the alcohol being necessary to produce a miscible liquid with the chloroform and the acid diazo reagent.

Barron (1926), by adding bilirubin in N/20 NaOH (buffered with phosphate buffer to pH 8.43) to serum, found that a direct reaction was obtained only if bilirubin levels reached 16 mg. per cent or more. Below this level the reaction was always indirect. He therefore assumes that the diazo reaction is dependent on quantity. He theorizes that bilirubin on being formed in the reticuloendothelial system

is promptly adsorbed on globulin and is thus protected from being excreted by the kidneys, from rapid oxidation and from reacting with diazo reagent. The direct reaction occurs whenever the surface tension of blood is lowered by substances excreted from the liver. So far it has not been possible to convert an indirect serum to a direct one by adding bile salts in vitro, except by the addition of very large amounts of salt. This does not eliminate it as a possibility, however, because entirely different conditions may operate in the body with enzymes serving to catalyze the reaction.

Ducci and Watson (1945) studied the reaction rate curves of icteric serum with diazo reagent and came to the conclusion that the biphasic nature of the curve indicated the presence of two different types of bilirubin which reacted at different rates. Klatskin and Drill (1950) point out, however, that the same type of biphasic curve can be obtained from pure crystalline bilirubin.

Opposed to the blood catalyst theory are those workers who believe that bilirubin is somehow combined with protein and that alcohol or alcohol groups are necessary to release this combination and permit coupling with diazo reagent. Van den Bergh himself (1921) was undecided between two possible explanations. He believed it was possible that normal circulating bilirubin (later called haemobilirubin) had a different composition from bilirubin which had passed through the liver cells (cholebilirubin). The other possi-

bility was that bilirubin could be present in blood either in the free state or linked with blood proteins or lipids.

Martin (1948) and also Gray and Kekwick (1948) have shown by electrophoresis that some bilirubin always travels with albumen and some with alpha globulin, no matter what the source of the serum. They conclude that the binding of bilirubin to constituents of plasma can have little to do with the direct diazo reaction.

Wunderley and Reynaud (1952) have found, by electrophoretic methods that bilirubin up to the amount of 5.7 mg. per cent attaches itself entirely to the albumen fraction of blood and travels with it. When the level of bilirubin is higher than this, "saturation" of the albumen fraction results in bilirubin becoming attached to globulin. By adding pure bilirubin to the three globulin fractions, they were able to show that the spectral absorption maxima are affected differently by the different fractions. The maxima are lowered most by the bilirubin being attached to alpha globulin and least by beta globulin with gamma globulin being midway between these two in its effect.

Lemberg and Wyndham (1936) have taken the view that the direct acting bilirubin is the one which is attached to protein and that the indirect fraction is not so attached.

In an effort to resolve all these various theories and to come to some understanding of the nature of the two reactions, Cole and Lathe (1953) tried to separate the bile

pigments of serum on a reverse phase chromatographic column. They made use of the information given by Hunter (1930), Sepulveda (1942) and others on the difference in solubility in chloroform of the two bilirubins. By chromatographic methods they have been able to separate from serum three fractions, two of which are much more polar in nature than the third, reacting directly with the diazo reagent. The least polar of them gives an indirect reaction and is believed to be bilirubin, because of its absorption spectra at mu 4500 which coincides with that of pure bilirubin. These fractions have apparently no protein left after being treated with alcohol and ammonium sulphate, to precipitate the protein, and the supernatant evaporated and applied to the column. Tests which would have revealed as little as 0.002 mg. protein in the eluate from these chromatographic columns have been entirely negative.

This method seemed worthy of further study and for that reason the present work was undertaken.

# PART II

## EXPERIMENTAL

### Details of the Method

Cole and Lathe (1950) took note of the work of Howard and Martin (1950) wherein a reverse phase chromatographic column was used in the separation of fatty acids. In this method a chromatographic column is made of non-wettable kieselguhr which supports a nonaqueous phase of a two phase system. The material to be separated is dissolved in the more aqueous and polar phase and applied to the column. As the two phases flow past one another a separation of pigments is achieved owing to the different solubilities of the constituents in aqueous solution. Fractions are collected and can be evaporated to dryness and the residue reconstituted with water or bovine albumen solution to give positive or negative diazo reactions.

### Preparation of the column

Kieselguhr was treated according to the directions given by Howard and Martin (1950). Hyflo Super Cel was dried at 110° in the oven and when cool, was allowed to stand in a desiccator containing dichloro dimethylsilane. The kieselguhr was then aerated in a fume chamber for twenty-four hours with frequent stirring. The material was then

removed and washed with methanol to a pH of 6.0. Finally it was dried in an oven to 110° and stored in an air tight container. The material floated when placed in a beaker of water.

The phases to be used on the column were prepared according to the method of Cole and Lathe (1953). The solvent system used contained chloroform 25 vols., carbon tetrachloride 25 vols., absolute methanol 38 vols. and 0.025 M phosphate buffer pH 6.0, 12 vols. The phosphate buffers were made according to the method of Hawk, Oser and Summersen (1949). These constituents were well shaken, stored overnight in a separatory funnel and separated. The bottom phase is the stationary, non-polar one and the top layer is the polar mobile phase.

The columns were made ready for use by mixing 6 gms. of kieselguhr with 3 ml. of stationary phase, 17 ml. of the mobile phase were added rapidly and the material tamped down by means of a stainless steel plunger. The column itself was a 1.8 cm. diameter glass tube, about 15 cm. in length and with a constriction at the bottom. The bottom of the column was plugged with cotton wool to hold the kieselguhr. (Figure 2).

The blood serum to be fractionated was freed of protein by treating 1 vol. of serum with 0.18 vols. saturated $(NH_4)_2SO_4$ and 2.5 vols. of 95% ethanol. This was shaken and allowed to stand one hour before centrifuging and decanting

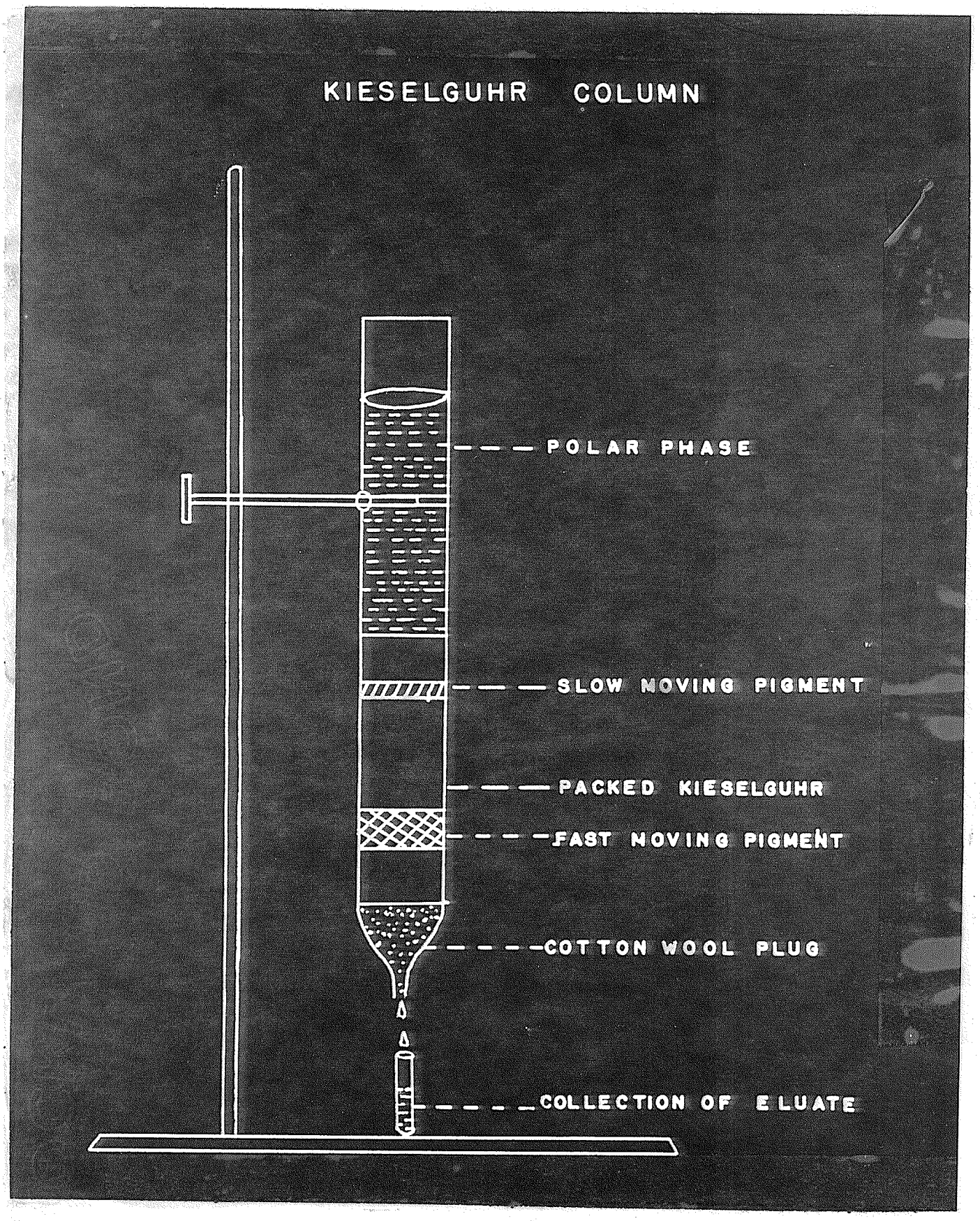


Figure 2

The Kieselguhr Column

the supernatant fluid. The fluid was then dried in vacuo at room temperature, and the residue was re-dissolved in 2 ml. of top phase and applied to the column. In the case of serum from cases of erythroblastosis, it was not possible to get the material into solution in the aqueous phase. In this case a small amount of the kieselguhr was added in alcohol, the material dried and taken up in the top phase.

All material being eluted from the column was collected in 1 ml. fractions. The speed of elution varied from column to column according to the amount of weight of fluid placed on top of it. The best rate of elution was about 1 ml. in four minutes.

The optical densities of the eluates were read with a violet filter, Kodak #1 (mu 543) in a Hilger Spekker Absorptiometer. The optical densities were then plotted against rate of elution as abscissae. In some cases the eluate was examined in a Uvispek spectrophotometer to determine the absorption spectra in ultra violet and visible light.

Some of the eluates were dried in a stream of air at reduced pressure, redissolved if possible in water and then used in the diazo reaction. After determining the diazo reaction in water solution, alcohol was added to make a final solution of 50% and the indirect diazo reaction was measured.

The diazo reaction technique was that of Malloy and Evelyn (1937), the direct action being measured at one minute

according to the modification of Ducci and Watson (1945).

The experimental work in the study of bilirubin can be divided into six sections. The following detail of the work is representative of the results obtained from 73 experiments. Of these 73, twenty have been selected for description and full explanation. Each graph represents the mean result of several experiments. The six sections are:

1. A study of the limitations of the technique with reference to the effect of the pH of the media, the presence or absence of protein and other factors.

2. The use of the column in assessing the methods for converting one fraction of bilirubin to another which have been suggested by various authors.

3. An attempt to discover substances in the blood which might be utilized in the transport of bilirubin in the body.

4. Experiments designed to show whether the method can be used to separate adult from fetal hemoglobins and bilirubins.

5. The separation of three pigments on a special butanol-water phase system.

6. The attempted production of kernicterus in newborn rats with a view to investigating the pigment in the brain tissue.

## SECTION I

### Experiment 1

A pure solution of bilirubin in chloroform containing 0.5 mg. of bilirubin was evaporated to dryness, then taken up in 2 drops of 2% $Na_2CO_3$. Material was then buffered to

pH 7.0 with 0.2 M Phosphate buffer of pH 6.0. It was then treated with 0.18 volumes of saturated ammonium sulphate and 2.5 volumes of 95% ethanol to parallel the technique for deproteinization.

The mixture was allowed to stand for one hour and was evaporated to dryness under reduced pressure at room temperature. The dry residue was taken up in 2 ml. of top polar phase and applied to the newly prepared column. When this material had entirely sunk into the kieselguhr, more of the polar phase was added to the top. As the bilirubin mixture moved down the column, two distinct bands of colour began to separate out.

As the eluate came off the column at the rate of about 1 ml / 4 minutes, it was collected in glass tubes in 1 ml. fractions. The optical density at mu 543 of each tube was measured. These optical densities were then plotted as ordinates on linear graph paper, and the number of ml. of eluate were plotted as abscissae.

The results of this separation are shown in Fig. 3.

Fig. 3 shows that even pure Hoffman La Roche bilirubin will separate into two distinct bands of colour when it is prepared in this way and put through a kieselguhr column. In a personal communication from G. H. Lathe, he attributes the appearance of two bands of colour from commercial bilirubin to the presence of impurities in the material.

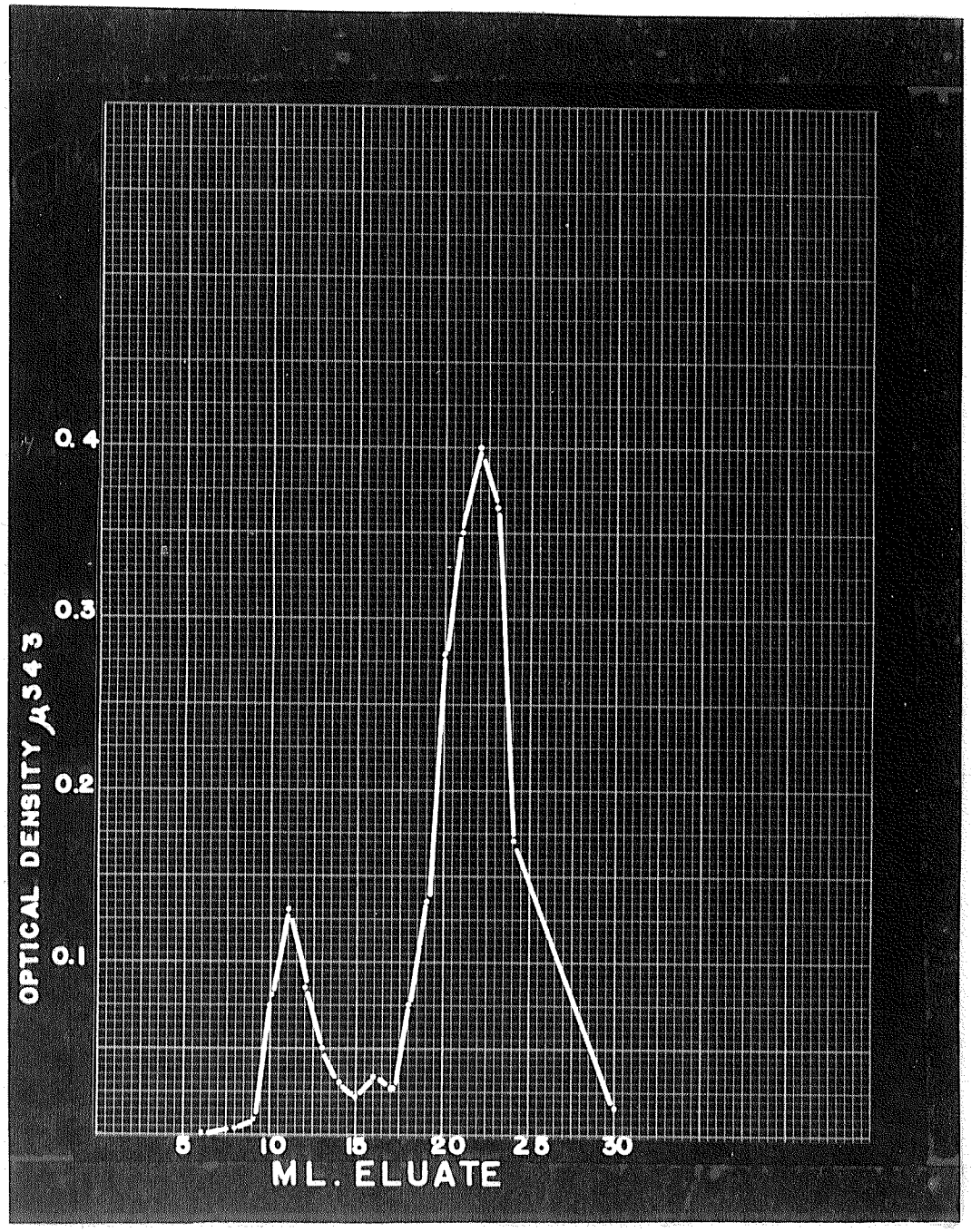


Figure 3

The Separation into Two Fractions of Pure Hoffman - La Roche Bilirubin

The two bands of colour which were obtained were examined spectroscopically to determine their absorption maxima. It was found that the first band of colour eluted from the column (the fast band) had an absorption maximum at 2750 A. The second and slower moving band was found to have its absorption maximum at 4500 A which is the accepted peak for pure bilirubin.

The two bands of colour in the eluate were evaporated to dryness and then the dried material was dissolved in water and the diazo test of Evelyn and Malloy (1937) was applied. The amount of material present was too small to determine whether the reaction was positive or not. A positive diazo test was obtained on the material from the slow moving, larger band of pigment only after 50% by volume of ethyl alcohol had been added.

## Experiment 2

This experiment was designed to show the effect of adding pure bilirubin to normal, non-icteric human serum.

A pure solution of bilirubin in chloroform containing 0.28 mg. bilirubin was evaporated to dryness. This was dissolved in 2 drops of 2% $Na_2CO_3$ and then added to 1.5 ml. normal serum. The final pH was 8.8. This preparation was then precipitated in the usual way with ammonium sulphate and alcohol. After centrifugation, the supernatant fluid was taken up in 2 ml. of top polar phase and applied to the

column.

Two bands of pigments immediately began to separate out on the column. The results of reading the optical densities at mu 543 are shown in Fig. 4. Once again the results show that although only bilirubin and normal serum have been added to the column, nevertheless two bands of pigment were eluted, and the fast moving band was shown to contain pigment which, on drying, could couple directly in aqueous solution. The slow band contained pigment which, on drying, could not be dissolved in water and which gave azobilirubin only after the addition of 50% by volume of alcohol.

The absorption spectra showed the slow band to have a maximum absorption about 4500 A while the fast band showed a sharp peak at 2850 A.

The relative quantities of fast and slow moving pigments did not appear to be appreciably altered by the addition of serum to the solution of pure bilirubin.

## Experiment 3

The previous experiment was repeated but with the alteration of the final pH of the solution of serum from pH 8.8 to pH 7.0.

A bilirubin in chloroform solution containing 0.28 mg. bilirubin was evaporated to dryness. The dry material was taken up in two drops of 2% $Na_2CO_3$ and then 1.5 ml. of normal, non-icteric serum was added. The solution was then

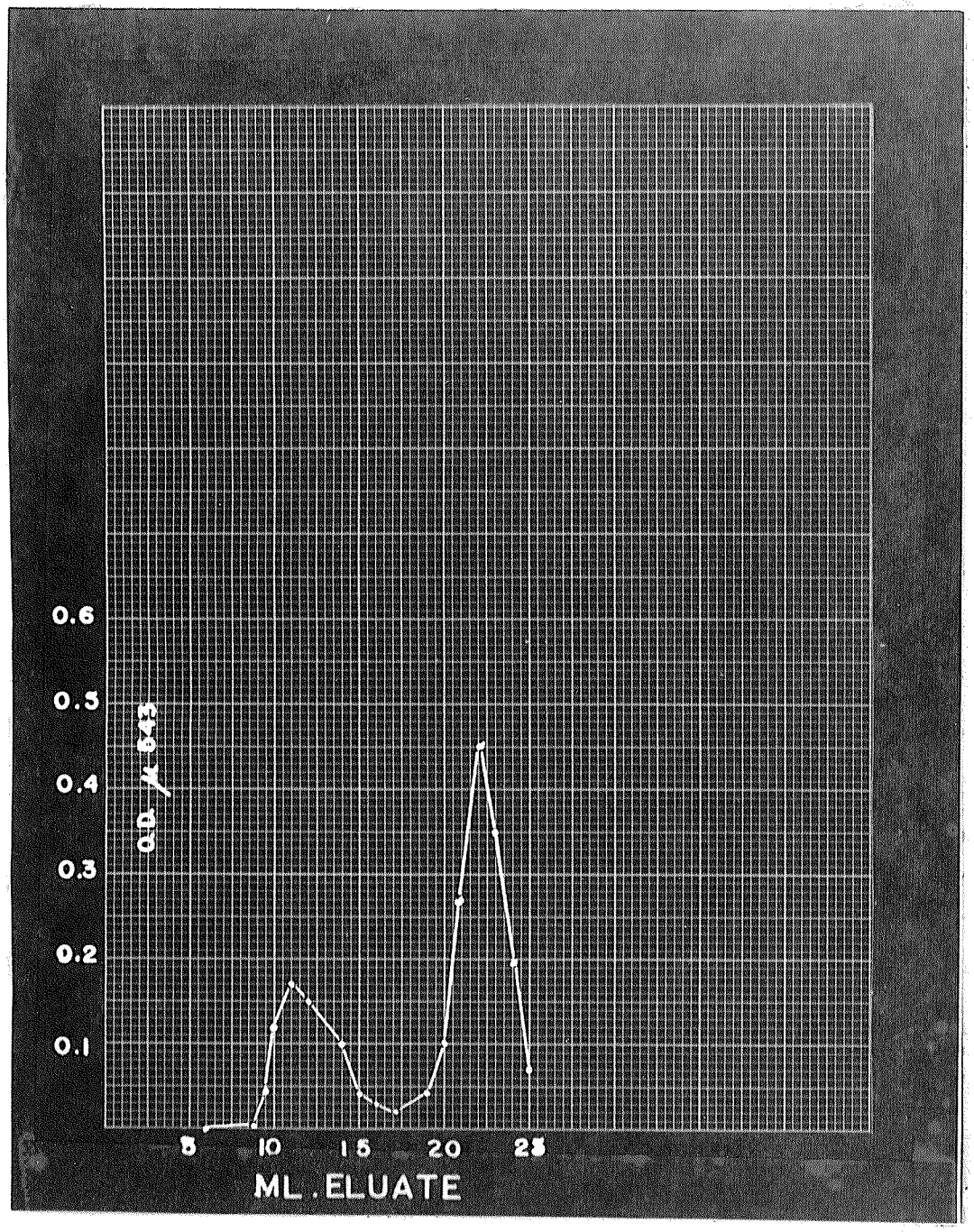


Figure 4

Addition of Pure Bilirubin to a Normal Non-icteric Blood Serum

buffered to pH 7.0 with 0.2 M phosphate buffer of pH 6.0. Ammonium sulphate and alcohol were used for precipitation of the proteins. After centrifuging, the supernatant fluid was dried off under reduced pressure at room temperature and the residue taken up in 2 ml. of top polar phase.

In this experiment three distinct bands of pigment appeared on the column. The most polar and rapidly moving band was eluted at about 12 ml. and a less polar one at 15 ml. and the least polar one at 20 ml. The middle band of pigment is likely the Pigment I described by Billing (1955). The small peak of colour was too faint to study the absorption spectra or to determine its reaction to the diazo reagent.

## Experiment 4

In an effort to try to obtain a larger middle band of colour which would be concentrated enough to study in the spectrophotometer, Experiment 3 was repeated. The amount of bilirubin used was doubled, and 0.5 mg. of bilirubin were applied to the column. The same method of preparation using sodium carbonate and normal serum was used. The pH was adjusted to 7.0 with phosphate buffer.

The middle band of colour which was to be studied failed to appear at all, as is shown in Fig. 5. The larger amount of bilirubin used caused the two major bands of pigment to trail one into the other, and obscure the presence

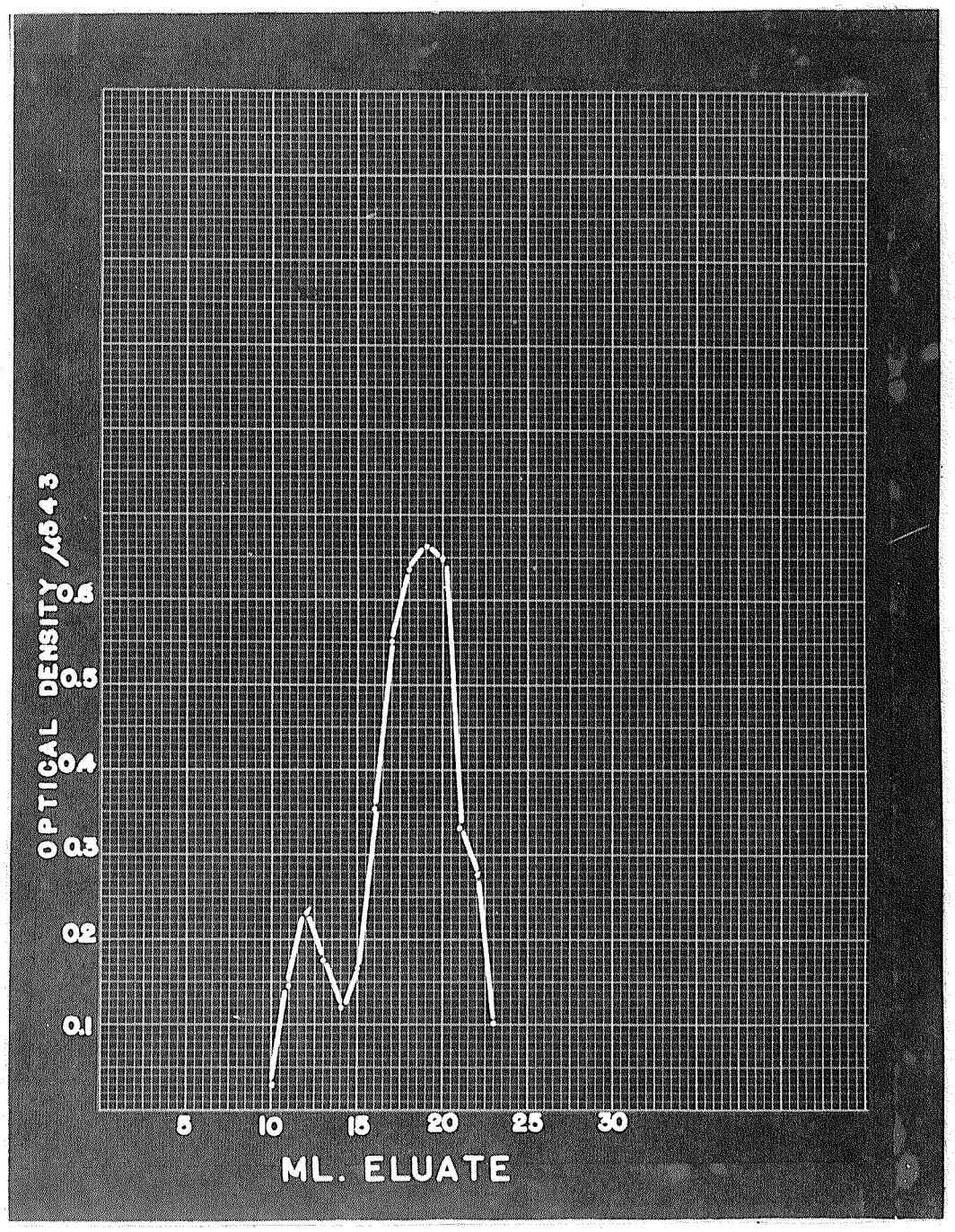


Figure 5

**The Effect of Overloading the Column with Excess Bilirubin**

of the middle band of pigment.

Fig. 5 shows the effect of using the larger amount of bilirubin on the column. Only two peaks were found, one at 12 ml. and the other major one at 19 ml. The fast moving band was collected together and the eluate dried off in a stream of air at reduced pressure. The dried residue was dissolved in water and gave a positive direct diazo reaction. The slow moving band when treated in the same way was not soluble and gave a positive diazo reaction only after a 50% volume of alcohol had been added.

## Experiment 5

It seemed obvious from previous experiments that the pH of the material being added to the column had a pronounced effect on the relative size and speed of elution of the pigment. To study this effect, two parallel columns were set up.

Two tubes were arranged similarly, with 0.5 mg. of bilirubin dissolved in two drops of sodium carbonate. To these were added 1.5 ml. of normal non-icteric serum. Two tubes were then treated with 0.2 M phosphate buffer of pH 6.0. One of the preparations was returned to pH 8.0 and the other to pH 7.0.

Each was then precipitated using ammonium sulphate and alcohol. After standing one hour, they were centrifuged and the supernatant fluid poured off and dried under reduced

pressure at room temperature.

The residue of each was taken up in 2 ml. polar phase and applied to two columns at the same time. Eluates were collected separately and the speed of the elution was plotted against the optical density measured at mu 543.

Fig. 6 shows the results of the above two preparations. It clearly indicates the effect of pH before deproteinization. At pH 8.0 much more pigment is eluted in the fast moving band than when the pH is 7.0. There is apparently no difference in the rate of elution of slow and fast moving pigment at 27 ml. Only the quantity of pigment in the fast moving band is different, almost twice as much appearing in the serum of pH 8.0 as in the serum of pH 7.0.

The eluate from the serum at pH 8.0 was collected and pooled. From the fast moving band a dry residue was obtained which could be dissolved in water to give a positive direct diazo reaction. The absorption spectrum of part of the fast moving pigment was determined on the Uvispek spectrophotometer. Fig. 7 shows the results of this examination. The peak of absorption is found in the ultraviolet region at 2750 A, and there is a plateau in the visible region which ends sharply at 4500 A. It is known that the absorption maximum of pure direct bilirubin crystals is at 4150 A according to Najjar (1955), and therefore this peak at 2750 A may possibly be due to the presence of small amounts of protein in the eluate of the fast moving band of pigment.

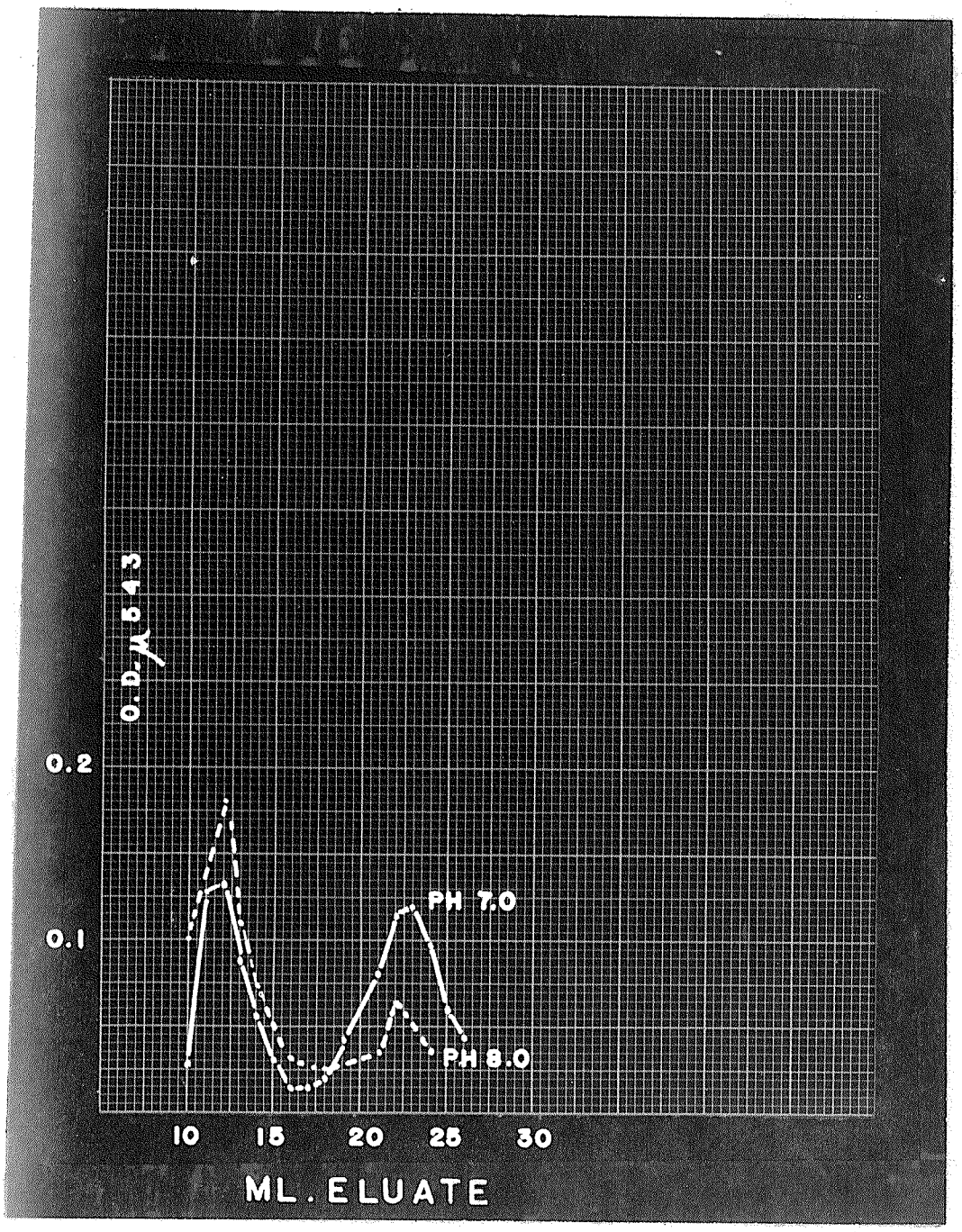


Figure 6

Material for Chromatography Prepared at pH 7.0 and at pH 8.0

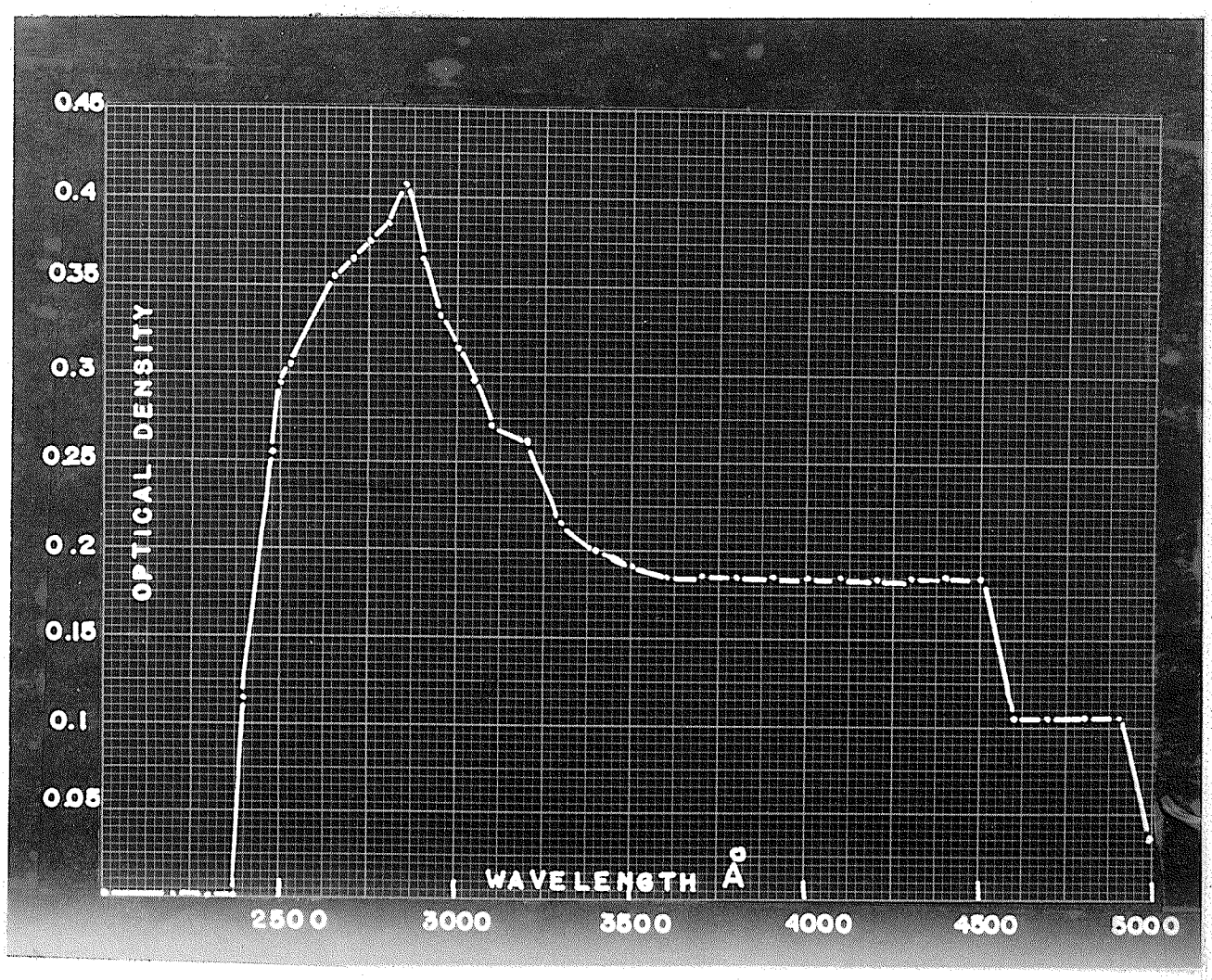


Figure 7

The Absorption Spectrum of the Fast Moving Band of Pigment from Material Prepared at pH 8.0

## Experiment 6

The behaviour on the column of serum known to contain predominantly indirect bilirubin was studied.

Serum from an erythroblastotic infant was obtained and treated with ammonium sulphate and alcohol to precipitate the proteins. The supernatant fluid was dried and the residue taken up in top polar phase. It was noticed that the residue was very poorly soluble in this medium and this again is in agreement with the observations of Hunter (1930) and Sepulveda (1942) and others on the solubilities in aqueous solution of direct and indirect bilirubin fractions.

Fig. 8 gives the result of the separation of this type of serum. Two bands of pigment were eluted from this sample of blood. Although the serum was known to give a very small direct diazo reaction, nevertheless, on the column, this was the predominating fraction. The reason for this is that the indirect acting pigment was only very slightly soluble in the polar phase which is aqueous.

## Experiment 7

In order to find out just how much pigment was being lost by not being dissolved in polar phase, the dried residue which had not gone into solution was saved and treated by shaking with ether. More pigment was seen to go into solution and the coloured ether was then poured off and evaporated to dryness. The dried residue, free of ether

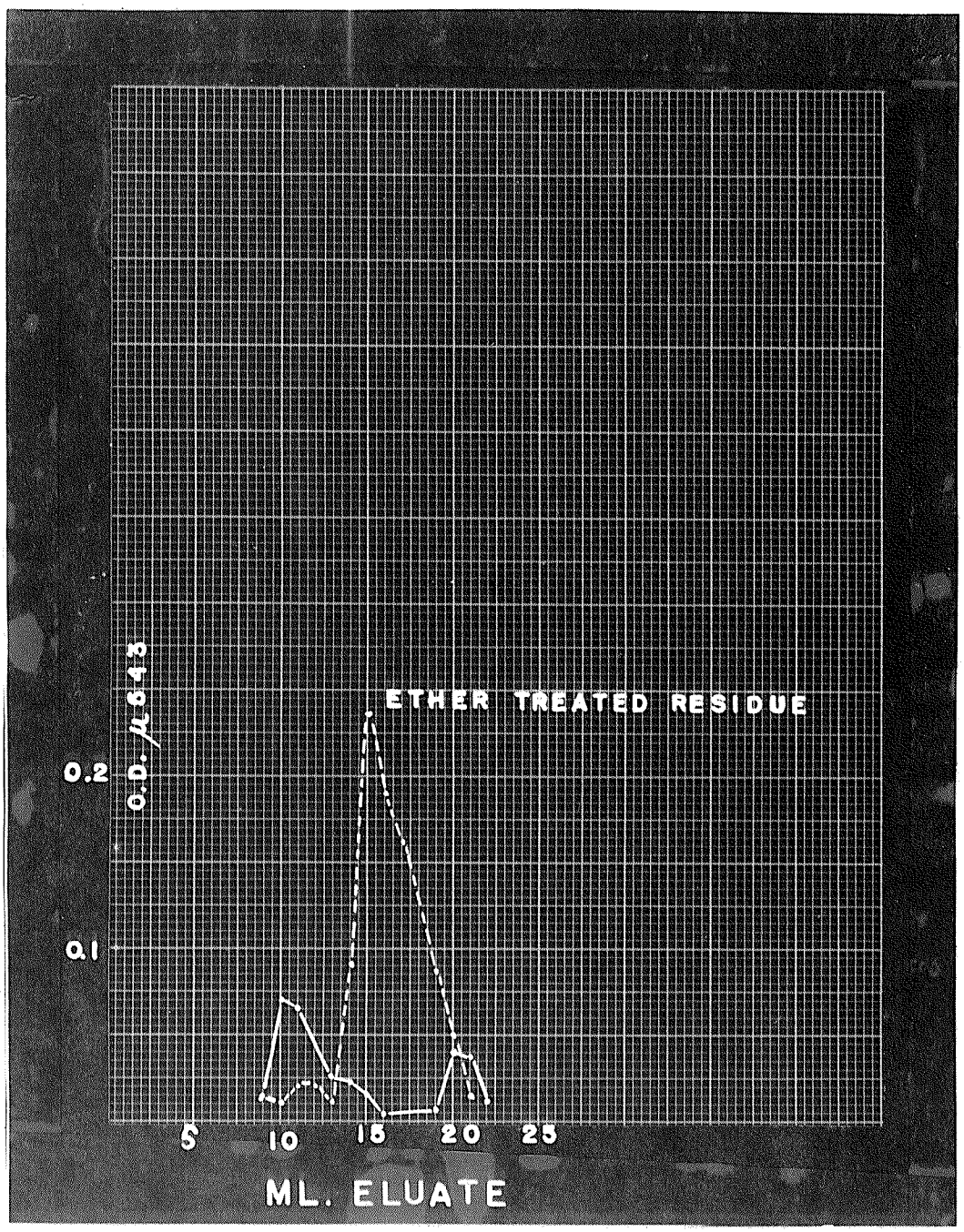


Figure 8

Blood Serum from an Erythroblastotic Infant, the Undissolved Residue Taken up in Ether

was then taken up in 2 ml. of polar phase and applied to a new column.

Fig. 8 shows, in the dotted line, the result of treating the undissolved pigment in the above described way. It can be seen that there is a very great loss of the indirect pigment when the method of preparation is that described by Cole and Lathe (1953). The fraction of pigment which was thus obtained was collected and the eluate dried off under reduced pressure. The dried pigment could be dissolved in a mixture of water and alcohol and then gave a positive diazo reaction.

Experiments 6 and 7 lead one to doubt the efficiency of this method of preparing material for chromatography. The polar phase is too aqueous a material to dissolve properly the pigment from the serum of an erthythroblastotic patient.

## Experiment 8

Because so much difficulty had been encountered in getting indirect pigment to dissolve in polar phase, several attempts were made to treat the residue containing pigment in various ways, in the hope of getting it all into solution.

Since the indirect pigment was the most difficult to get into solution, erythroblastotic serum was used. One ml. of highly icteric serum was deproteinized using ammonium sulphate and alcohol. The mixture was allowed to stand one hour, then centrifuged and the supernatant fluid removed.

This was dried in vacuo and taken up in polar phase and applied directly to a column. A large amount of material did not go into solution and this was dissolved in chloroform, which was poured off and evaporated to dryness in the same way as ether had been used before. This dry material was then able to dissolve in top phase and was applied to a second column at the same time as the other preparation without chloroform.

The eluates from each column were collected and the speeds plotted as abscissae, with optical density at mu 543 as ordinates. Both were plotted on the same graph for comparison.

It can be seen from Fig. 9 that only about one quarter of the total pigment went into solution in top phase. Chloroform brought the remaining pigment into solution and the peak of elution was at 18 ml. Almost all the direct acting material went into solution in the aqueous phase, but a small amount remained to be dissolved by chloroform.

The solid line (A) in Fig. 9 is the plot of the material obtained without the use of chloroform, while the dotted line (B) of Fig. 9 shows the result of the chloroform treatment of the undissolved residue.

According to the absorption spectra, the material dissolved in top phase and that which dissolved in chloroform were both bilirubin. The absorption maxima in both cases was at 4500 A. The maximum of the small, fast moving band of

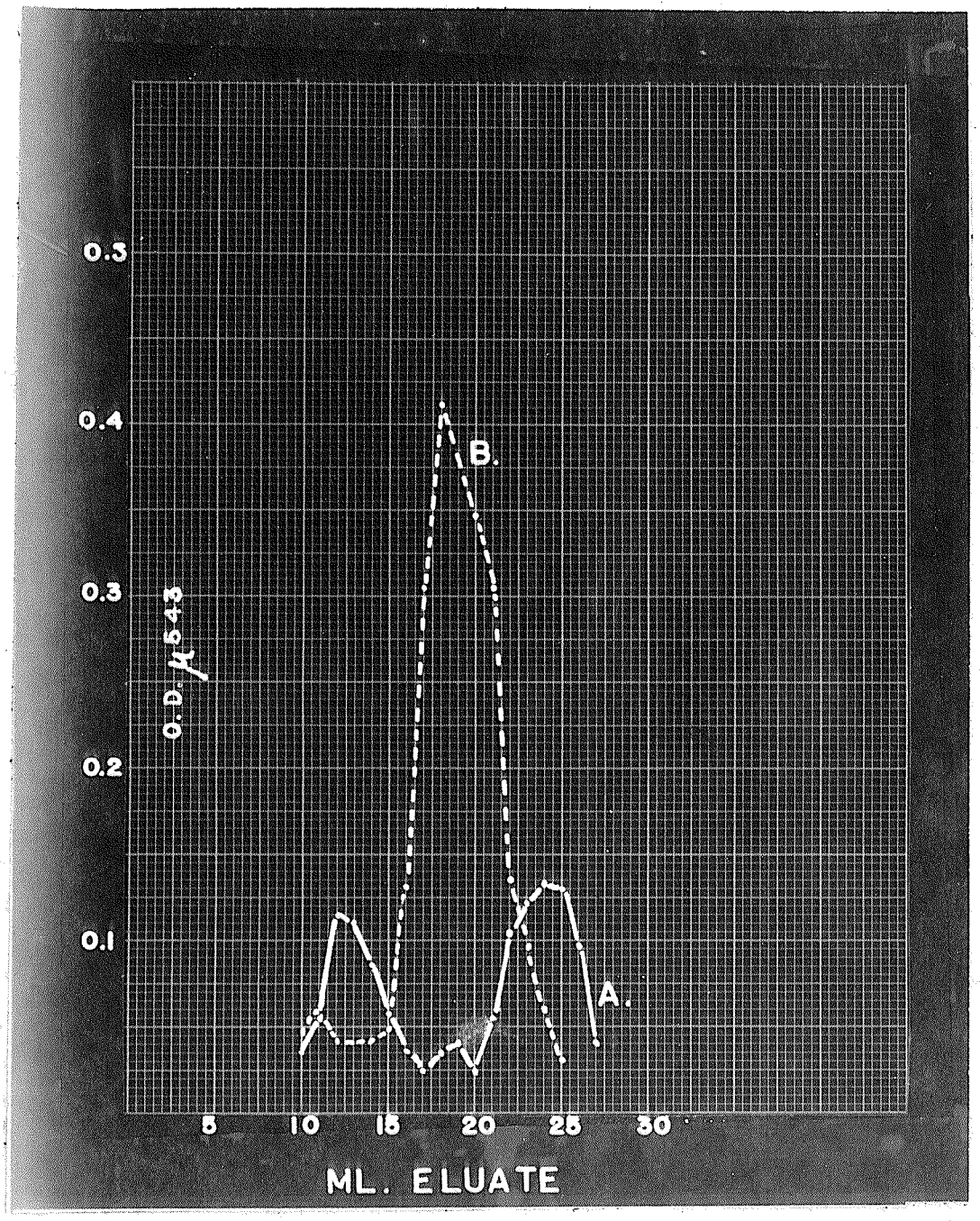


Figure 9

Blood Serum from an Erythroblastotic Infant, the Undissolved Residue Taken up in Chloroform

pigment was at 2900 A. The two bands of bilirubin came off the column at different speeds. The chloroform soluble fraction was eluted at 18 ml. while the fraction which was freely soluble in top phase was eluted at 25 ml.

There is no satisfactory explanation of this difference. Possibly, chloroform has dissolved some material which is attached to bilirubin pigment and which serves to slow its progress on the column.

## Experiment 9

In a recent publication, Billing (1955) has taken note of the difficulty in dissolving indirect bilirubin fractions in polar phase and has corrected her technique of preparation of material for chromatography. She has suggested that, before the supernatant fluid from the deproteinization is dried completely, a small amount of dry kieselguhr in alcohol be added. The indirect pigment is adsorbed to the kieselguhr and the alcohol is then evaporated off. Polar phase will then take up nearly all the pigment from an indirect acting serum.

This method of preparation was utilized in the preparation of a serum from a case of infectious hepatitis. In this case, 1.5 ml. of serum was treated with 0.28 vols. of saturated ammonium sulphate and 2.5 vols. of 95% ethanol to precipitate the proteins. The supernatant fluid from this was dried and before it was completely dry, a pinch of

dry kieselguhr in alcohol was added. The pigment was seen to adhere to the powder. The small amount of alcohol was then evaporated off, and the residue was taken up readily in polar phase and applied to the column.

Fig. 10 shows the result of this treatment. Three distinct bands of pigment were observed, the slow moving peak being the major one with a peak of elution at 28 mm. It is evident from the large amount of pigment in this slow moving band that the solution in top polar phase has been greatly enhanced by the treatment with kieselguhr.

## Experiment 10

It had been noticed that a direct acting serum, such as the serum from a case of obstructive jaundice, always showed a large proportion of pigment adhering to the protein precipitate after the deproteinization process. There seemed to be a definite affinity of this direct acting pigment for protein. This is in agreement with the findings of Hunter (1930) who summarized the differing properties of direct and indirect bilirubin and who mentioned that direct bilirubin is brought down in protein precipitation with alcohol. That most of the direct acting bilirubin is lost in using the chromatographic method of Cole and Lathe was proved by treating the protein precipitate with an aqueous trypsin mixture.

One millilitre of a saturated solution of trypsin was added to the protein precipitate from a case of obstructive

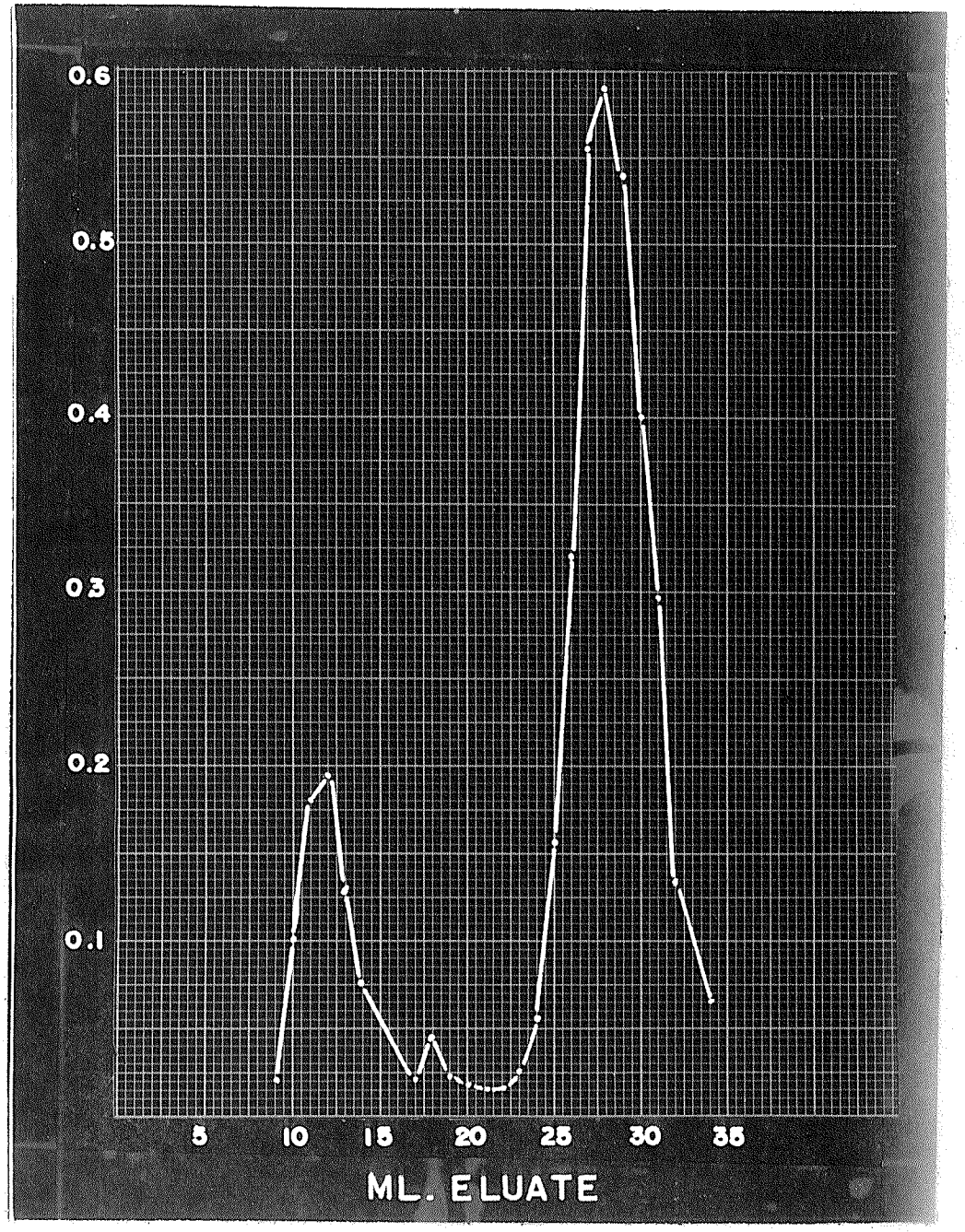


Figure 10

Blood Serum from a Case of Infectious Hepatitis
Showing Three Distinct Bands of Pigment

jaundice. The precipitate was well stirred with the trypsin and placed in the incubator at 37° C. for three hours. At the end of this time the centrifuge tube and contents were spun down and the supernatant fluid noted. The trypsin solution was now strongly coloured with pigment. The trypsin had apparently detached the bilirubin pigment from the precipitated proteins. It was expected that direct acting bilirubin which was incubated for this long time would have been oxidized to biliverdin, but this did not apparently take place, since the solution remained yellow with no evidence of green. The bilirubin was somehow protected from ready oxidation by its attachment to the precipitated serum proteins.

The results of Experiments 8, 9 and 10 show that the chromatographic method as outlined by Cole and Lathe is by no means a quantitative one. It has a useful purpose in the identification of the fractions of bilirubin, and for their separation, but cannot be used to determine their relative amounts.

## SECTION II

Attempts were made to convert direct acting bilirubin to indirect using methods advocated by various authors.

### Experiment 11

Foweather (1932) believed that the direct bilirubin

is an alkali salt, probably the ammonium salt, but that it could be the sodium salt. He claims to have been able to convert an indirect bilirubin to a direct form, by dissolving bilirubin in a dilute alkali and precipitating direct bilirubin out by the addition of a concentrated solution of the same alkali.

This experiment was set up to see whether this method of Foweather's would produce a direct bilirubin which could be separated and identified, using the chromatographic column.

One mg. of dry bilirubin (Hoffman-LaRoche) was placed in each of two tubes, A and B. Tube B was treated by adding two drops of 2% sodium carbonate and then adding 1.5 ml. of non-icteric serum and buffering to pH 7.0 with phosphate buffer. Material in tube A was dissolved in 1 ml. dilute sodium bicarbonate solution. Then a concentrated solution of the same salt was added to precipitate the bilirubin again. Some flocculation did appear and this was allowed to accumulate and settle to the bottom of the tube. After centrifuging, the supernatant fluid was carefully removed. Obviously a great deal of pigment was removed as well, since the supernatant fluid was highly coloured. The remaining material was dissolved in 1.5 ml. of non-icteric serum. The pH at this time was 7.0 and the bilirubin appeared to be well in solution. Both tubes A and B were then treated to identical precipitation procedures. Supernatant fluids were

dried and taken up in top phase and placed on similarly prepared columns. The eluates were collected at the same time in an effort to avoid any difference due to change in temperature or other factors.

The result of this attempt at conversion of indirect bilirubin to direct form is shown in Fig. 11. There has apparently been no conversion of indirect pigment to direct. The dotted line shows the eluate coming from the sample of bilirubin treated in the usual way, whereas the solid line indicates the eluates coming from the bilirubin precipitated from weak alkali by a strong solution. There is actually relatively less of the fast moving band of pigment coming off in the alkali treated sample, even making allowances for the overall loss of pigment due to the manipulations.

This result was confirmed by repeating the first part of the experiment, and dissolving bilirubin in dilute sodium bicarbonate and then precipitating it with strong sodium carbonate. The material thus obtained could be dissolved in aqueous solution, but did not give a positive diazo reaction. On addition of alcohol, a positive diazo test was obtained. Thus it would appear that this method of converting one pigment to another does not prove to be possible, at least as demonstrated by chromatography.

## Experiment 12

Najjar (1952) also claims to have been able to con-

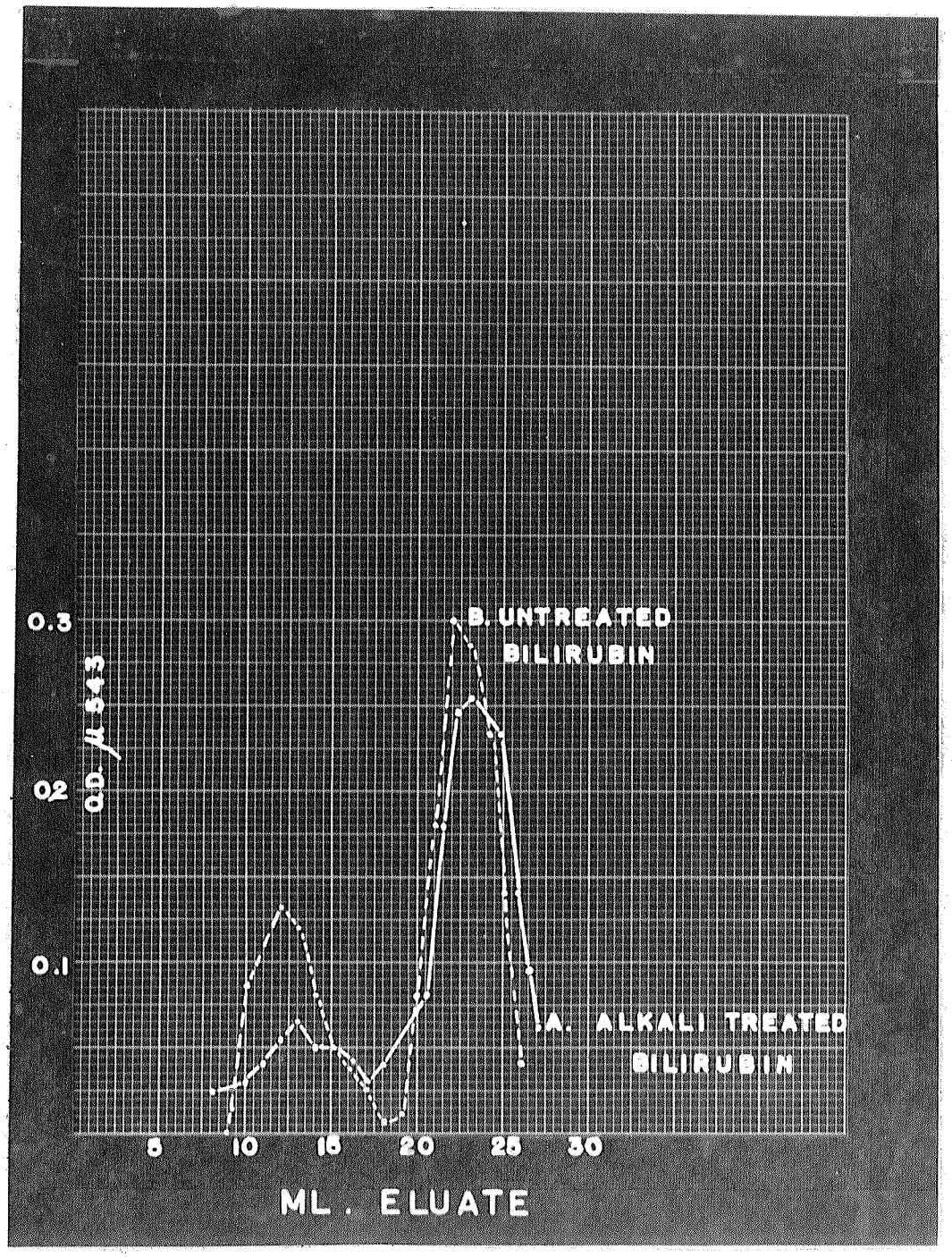


Figure 11

An Attempt to Convert Indirect Bilirubin to Direct Through the Formation of its Alkaline Salt

vert indirect bilirubin to the direct form by treating it with alkaline buffers of pH 10. According to Najjar, keeping indirect acting bilirubin in pyrophosphate buffer at pH 10 causes it to become direct acting.

This experiment was to demonstrate this conversion. 0.5 mg. bilirubin were placed in each of two centrifuge tubes. To tube A were added two drops of 2% sodium carbonate to bring the bilirubin into solution. 0.02 M phosphate buffer was added to bring the solution back to pH 7.0. This was kept overnight in darkness, and because oxidation often takes place, a crystal of dithionite was added to prevent the formation of biliverdin. The material was then precipitated by ammonium sulphate and alcohol and the supernatant fluid dried at room temperature and taken up in 2 ml. of polar phase.

Tube B was treated with Najjar's 0.01 M sodium pyrophosphate buffers and brought to pH 10.5 with 0.2 N NaOH. Three ml. of this buffer were added to the 0.5 mg. of bilirubin and a crystal of dithionite added. This was left overnight in the dark and then the proteins were precipitated. The supernatant fluid was dried and the residue taken up in 2 ml. of polar phase. The two tubes of material, A and B, were then applied to two columns at the same time.

Fig. 12 shows the results of this treatment. It is evident that although treatment with alkali has made a difference in the separation of pigments, nevertheless there

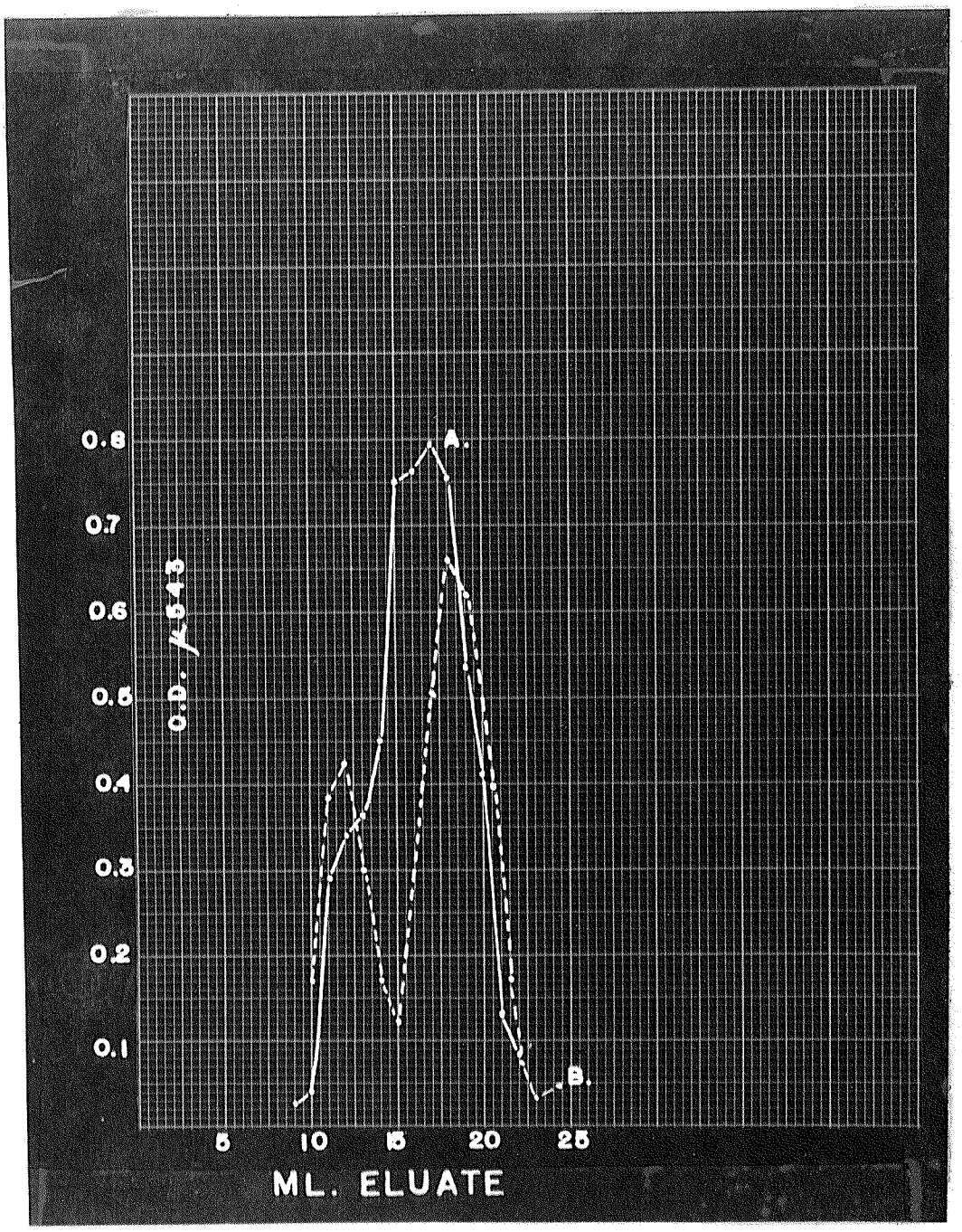


Figure 12

Crystalline Bilirubin Treated with Najjar's Phosphate Buffer at pH 10.0

can have been no real transfer from an indirect pigment to a direct one. The appearance of the two bands would lead one to believe that a new type of pigment had been formed. However, on evaporating the eluate from the fast band, it was found to be insoluble in water and did not give a diazo reaction either in water or in alcohol. It is likely that the alkalinity of the material is responsible for the rapid movement of some of the pigment. It is not likely to be the result of reduction by dithionite, because the control column treated similarly displayed only one band of pigment. The high alkalinity of the material applied to the column would doubtless prevent the diazo reaction in the eluate, since this reaction only takes place in acid solution. There is at any rate no proof of a change in pigment from an indirect acting to a direct acting one.

## Experiment 13

Victor Najjar (1955) has suggested that direct acting bilirubin was a bilirubin-metal-chelate complex in the alkaline medium which obtains in the bile. When the pH is lowered this complex can dissociate, but it is possible to render it more stable by its attachment to protein in a ternary complex. According to this theory, it should be possible to destroy this complex by treating the ternary complex with a metal chelating agent and thus destroy the complex. Najjar suggested that ethylene diamine tetra acetic

acid or perhaps cysteine might accomplish this.

In this experiment an attempt was made to convert a direct acting serum to an indirect serum by treating the serum with a chelating agent, cysteine.

1.5 ml. of serum from a case of obstructive jaundice known to be largely direct acting was treated with a molar solution of cysteine hydrochloride. The salt brought the pH down to about pH 2.0. The pH was adjusted to 7.0 by the addition of a few drops of 2% sodium carbonate. The material was precipitated with ammonium sulphate and alcohol. The supernatant fluid was taken up in 2 ml. of top phase and left overnight in the icebox.

No bilirubin appeared in the column; evidently the cysteine had reduced bilirubin to mesobilirubinogen, which is colourless.

## Experiment 14

Instead of trying to destroy a possible direct bilirubin-metal complex, it was decided to try to create such a complex by using some ion which might possibly attach itself to the bilirubin molecule and thus convert an indirect bilirubin to a direct one.

A sample of serum from an erythroblastotic infant was set up in the usual way in duplicate tubes. Precipitation of the proteins was carried out in the usual way using saturated ammonium sulphate and alcohol. However the alcohol

used for one of the tubes was saturated with sodium cyanide. The same volume of alcohol was used for precipitation in both cases. Then two parallel columns were run at the same time. The column to which the cyanide treated serum had been added immediately showed a strong band of colour which was eluted at the same speed as is direct bilirubin on an ordinary column. This very strong band of colour was eluted and the fractions from two or three tubes were pooled. This was then evaporated and the dry residue was tested for the diazo reaction. The residue was found to be readily soluble in water and to have a positive direct diazo reaction and, in fact, to behave in much the same way as direct acting bilirubin from any case of obstructive jaundice. Probably the sodium cyanide has formed a sodium bilirubinate which is soluble in water, and the high alkalinity of the material has accounted for its rapid progress through the column of kieselguhr. Fig. 13 shows the result of treatment with sodium cyanide. The solid line gives the amount of direct acting pigment, while the dotted line indicates the course of the untreated erythroblastotic serum.

## Experiment 15

Another possibility of converting indirect bilirubin to direct seemed to be by using the natural enzyme systems present in the fresh liver slices of rat.

In the controversy over the bilirubin-metal complex

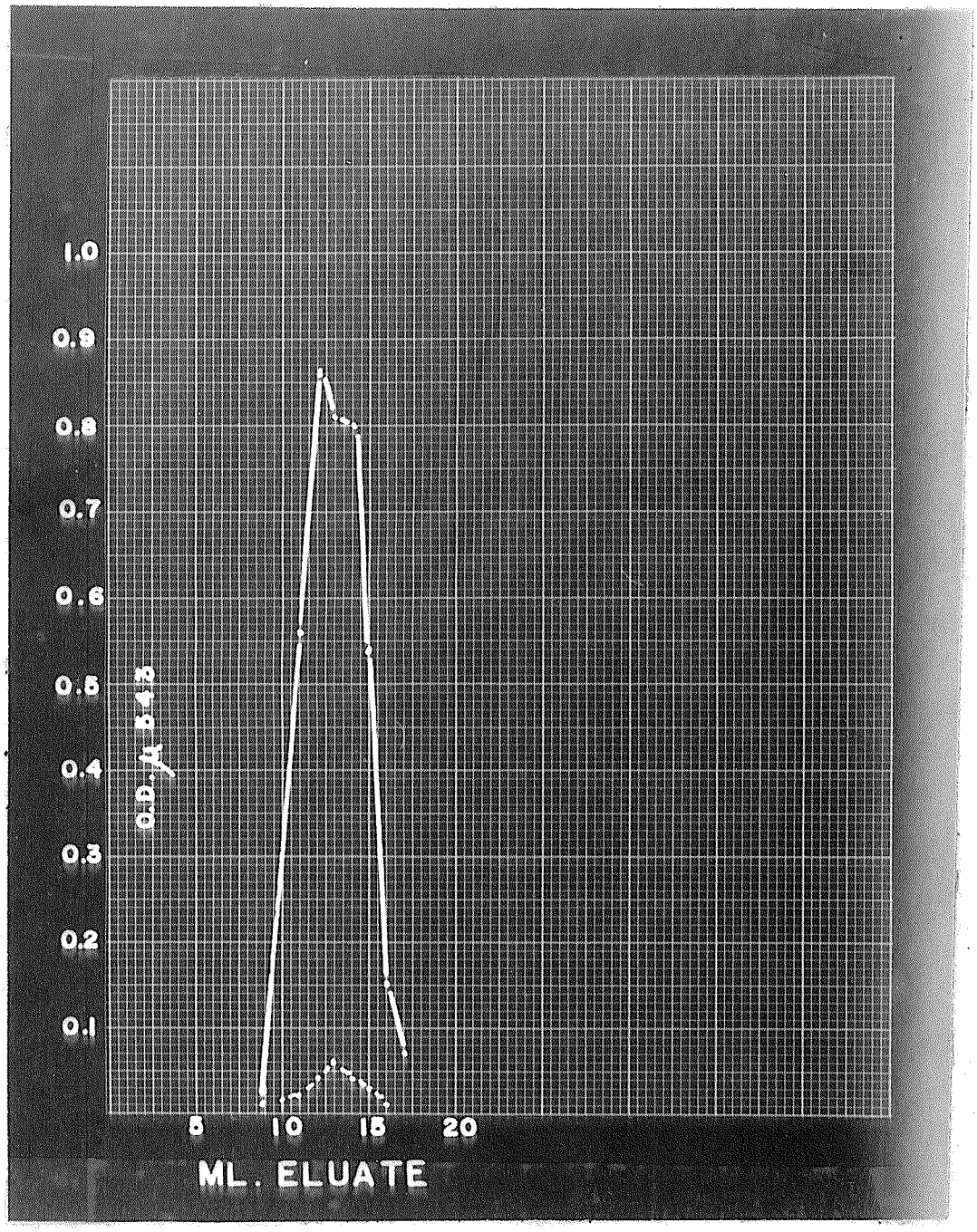


Figure 13

Indirect Acting Bilirubin Precipitated Using Alcoholic Sodium Cyanide

theory it seemed more important to try to convert indirect to direct serum than vice versa, because it is well known that direct serum which is allowed to stand at room temperature and exposed to the air will lose its direct action.

1.5 ml. of serum from an erythroblastotic infant was incubated overnight with slices of fresh liver from a full grown rat. The mixture was centrifuged and the supernatant fluid was deproteinized with ammonium sulphate and alcohol. The dried residue remaining after centrifugation and drying was then taken up in top phase. The material was applied to a column along with another column to which had been added the same serum, but without the incubation with liver. The result of the treatment with liver was to slightly increase the amount of direct acting pigment and to retard the speed of the slow moving, indirect acting pigment. The results were not definite enough to give any clear conclusion about the effect of the rat liver slices on the type of bilirubin.

## SECTION III

A study was made of the possible substances in the blood which might be involved in the transport of bilirubin in the blood.

### Experiment 16

Van den Bergh (1923) has suggested that indirect acting bilirubin may be attached somehow to a blood protein

or to a lipid.

This experiment was designed to show whether the addition of a lipid to pure pigment would cause a change in its speed of elution.

The lipid chosen was 90% pure lecithin (Nutritional Biochemicals) which was not further purified. A small amount (1 gm.) of the lecithin was added to 3 ml. of a 14 mg. per cent solution of bilirubin in chloroform. When lecithin was all in solution, the whole was poured off and dried under reduced pressure at room temperature. Complete drying was not possible, the residue being a frothy, gummy mass. To this was added 1.5 ml. of normal, non-icteric serum. It was then centrifuged and the liquid part removed from solid as well as possible with a dropper. About 1 ml. fluid was obtained. Deproteinization was carried out with 0.18 vols. of ammonium sulphate and 215 vols. of alcohol. This was allowed to stand for one hour and then centrifuged and the supernatant fluid dried at reduced pressure at room temperature. The residue was taken up in 2 ml. polar phase and applied to the chromatography column.

Fig. 14 shows the effect of adding lecithin to a solution of pure bilirubin. The most noticeable feature is the great slowing of the rate of elution of the second band of pigment. When no lecithin is added, this band of pigment is eluted at about 18-20 ml. Lecithin has had the effect of retarding this pigment so that it is eluted at 41-43 ml.

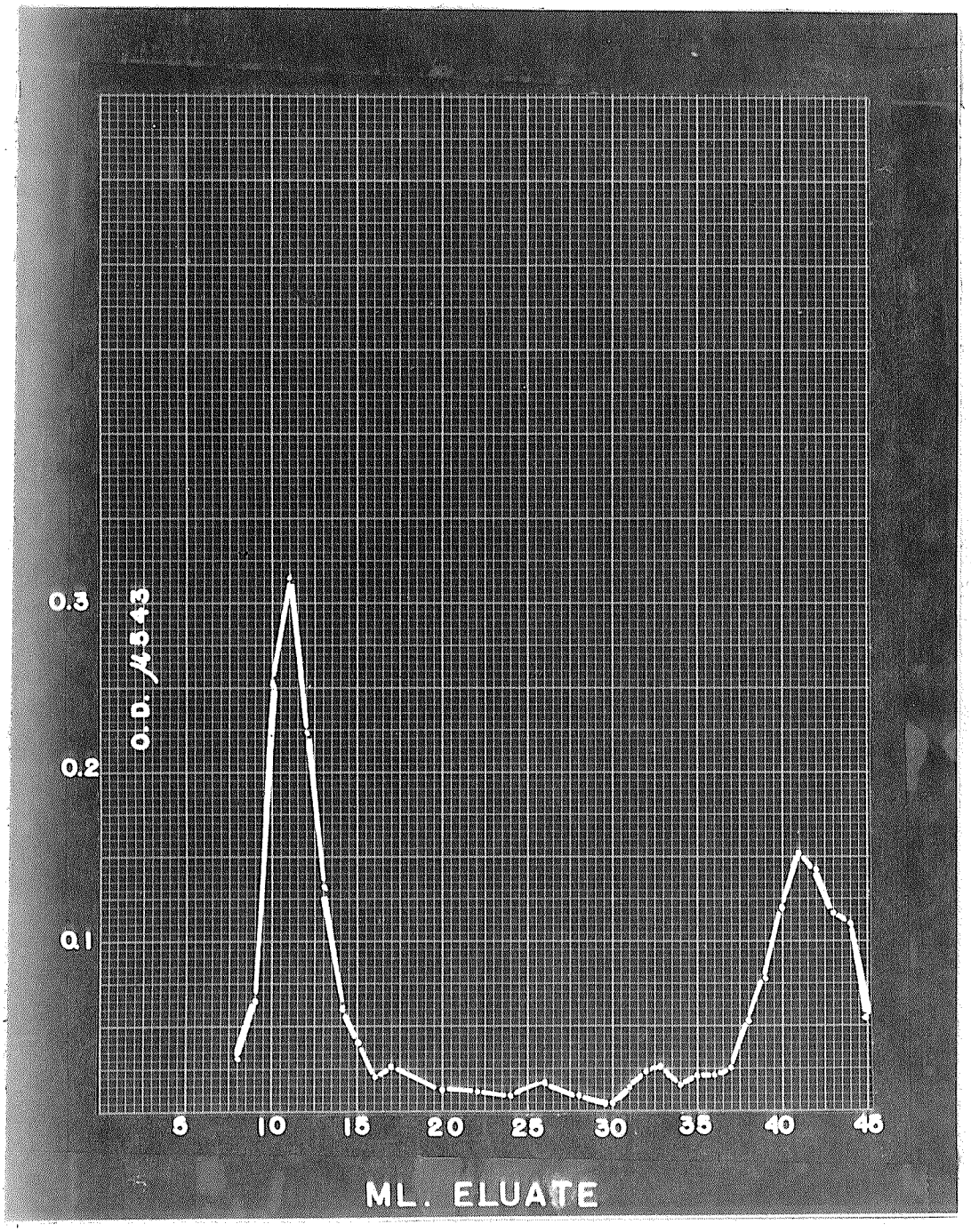


Figure 14

The Effect of Adding Lecithin to a Solution of Pure Bilirubin

The faster moving band of pigment was collected and the eluate dried. The residue was soluble in water but gave a negative diazo reaction. The slower moving pigment was also collected and dried. This residue was not soluble in water and even on addition of alcohol in the usual amount it did not give a positive diazo reaction.

The presence of lecithin has prevented the reaction with diazo reagent and retarded the rate of flow of bilirubin pigments.

## Experiment 17

Barron (1931) suggests that the direct reaction of serum occurs whenever the surface tension of blood is lowered by the secretion of substances from the liver.

The present experiment was carried out with a view to determining whether the addition of bile salt in vitro could cause a change in the diazo reaction of serum from indirect to direct.

About 500 mg. Na glychocholate was added to a bilirubin-chloroform solution containing 0.5 mg. bilirubin. The mixture was filtered and chloroform dried off. 1.5 ml. of non-icteric normal serum was added to the residue. The dried bilirubin appeared to go into solution in the serum. Deproteinization of the serum was carried out as usual. After standing an hour the supernatant fluid was poured off and dried under reduced pressure.

The eluates from the column were collected and optical densities at mu 543 were plotted as ordinates and the speed of elution as abscissae. Fig. 15 shows that the pure bilirubin which was added to non-icteric serum has resulted in two bands of pigment being separated on the column.

When the experiment was repeated using glycocholic acid instead of sodium glycocholate, only one band of pigment appeared on the column. It seems likely, therefore, that the sodium of the added salt has formed the salt of bilirubin in the form of sodium bilirubinate. This is known to be soluble in aqueous solution and in serum. The appearance of a fast moving band of pigment cannot be attributed to the formation of direct acting bilirubin. Any bilirubin-salt formation will cause bilirubin to be soluble in aqueous solution and therefore to move with polar phase.

## SECTION IV

Cole and Lathe (1953) gave results on the fractionation of serum bilirubins which seemed to indicate that indirect acting bilirubin from a case of haemolytic jaundice could be separated from indirect bilirubin from a case of obstructive jaundice. In the case of haemolytic disease the indirect, slow moving pigment was eluted from a kieselguhr column at a peak of 55 ml. The same type of pigment when obtained from a case of obstructive jaundice was shown to be eluted at 35 ml. This chromatographic method seemed to show

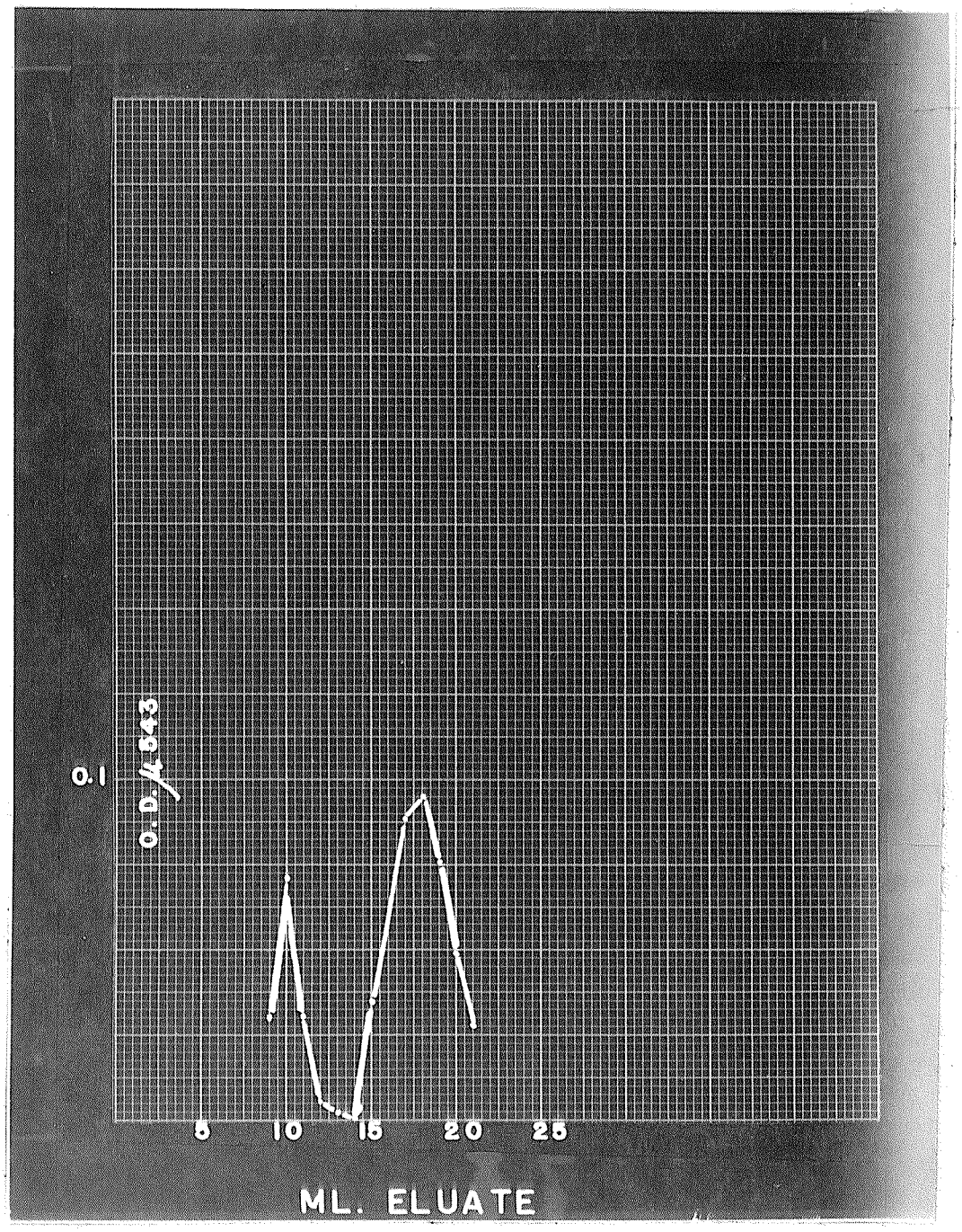


Figure 15

Na Glychocholate Is Used to Bring Bilirubin into Solution in Normal Non-icteric Serum

promise of being useful in the separation of two types of indirect acting bilirubin and from this it might be possible to guess at some of the differences in adult and fetal haemoglobins.

The fractionation of an erythroblastotic serum was already known from previous experiments, but chromatographic separation of serum from obstructive jaundice had not been done. It was necessary to know what the typical fractions of pigment were in these cases before determining whether there was any difference between adult and fetal bilirubin.

## Experiment 18

The behavior of a serum from a case of obstructive jaundice was studied on the kieselguhr column.

The material was prepared in the usual way and taken up in 2 ml. of top polar phase. The serum was known to be largely direct acting with the diazo reagent and it was noticed that much of the pigment adhered to the precipitated protein.

Fig. 16 shows the result of the separation of serum from obstructive jaundice. It can be seen that the major band of pigment appears as a fast moving fraction with a peak at 13 ml. The slow band was eluted at 21 ml.

The eluate from the fast band was dried and the residue dissolved in water easily. It then gave a positive, direct diazo reaction.

The eluate from the slow moving band gave a positive

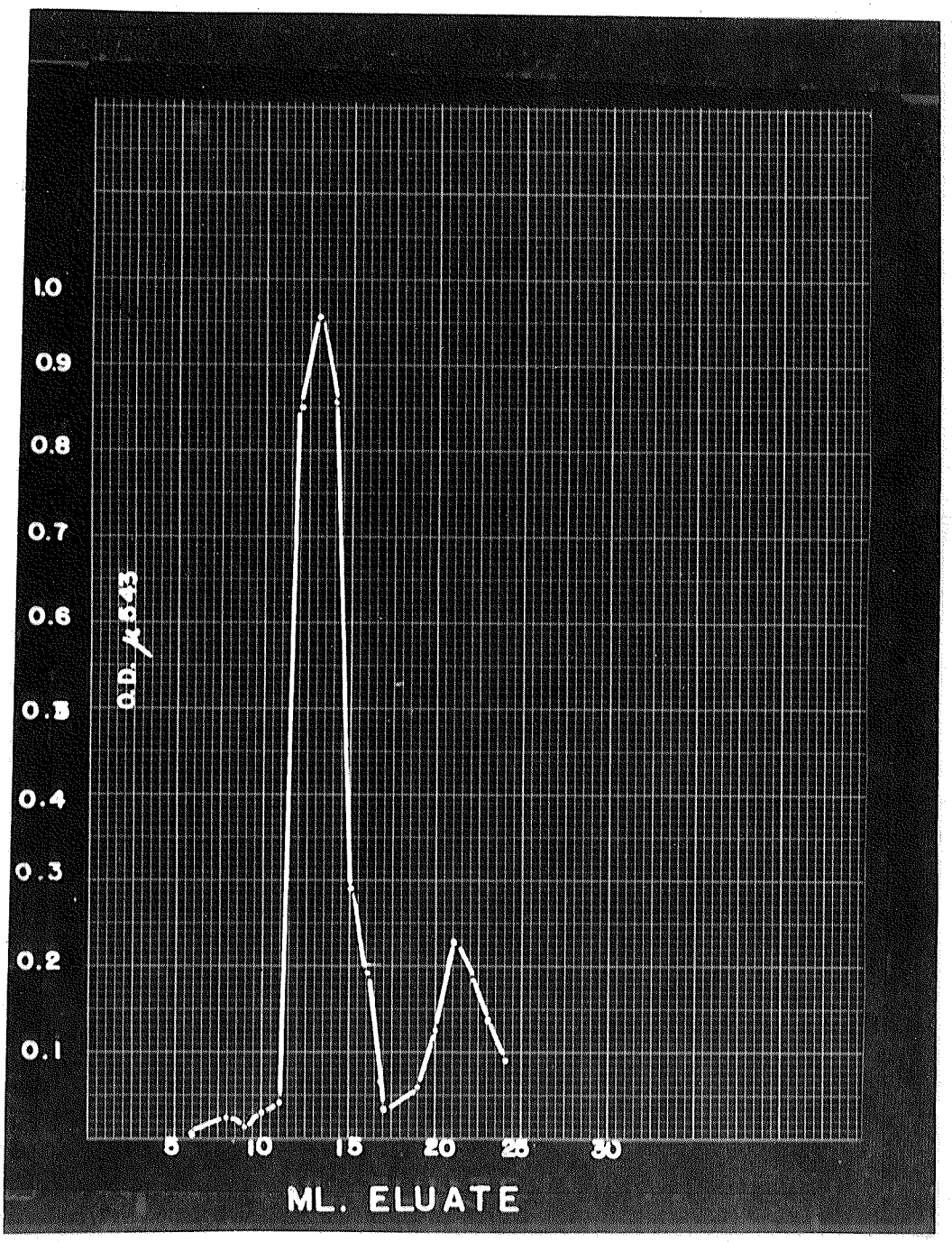


Figure 16

Blood Serum from a Case of Obstructive Jaundice

diazo reaction after the addition of alcohol.

## Experiment 19

In order to check the result of Cole and Lathe wherein they were able to obtain what seemed to be two different indirect bilirubin pigments, it seemed advisable to try to run two serums at the same time.

Serum from cases of obstructive jaundice and from a haemolytic jaundice were fractionated on the same chromatographic column.

0.75 ml. serum from each type of case were mixed together.

This 1.5 ml. of mixed serum was then treated with 0.28 vols. saturated ammonium sulphate and 2.5 vols. of 95% ethanol. The proteins were centrifuged and the supernatant fluid poured off and evaporated to dryness.

The resulting dry residue was taken up in 2 ml. of top phase and applied to a column.

Had there been two different types of indirect bilirubin present, two slow bands of colour would have appeared.

Fig. 17 shows that only one fast band and one slow moving band of pigment were eluted.

Cole and Lathe, in getting different speeds of elution for different fractions, must have used separate columns whose physical characteristics were slightly different. Differences in packing will cause changes in the rate of flow

of the pigments over the solvent.

Changes of temperature have been found to seriously affect the speed of elution and in fact a 10°F drop in temperature can slow the speed by one half.

It is concluded, therefore, that under exactly parallel conditions it is not possible to demonstrate any difference in indirect acting bilirubin from an adult case of obstructive jaundice and from a case of haemolytic icterus.

## SECTION V

Billing (1955) described a new phase system employing butanol-water, which could be used on a kieselguhr column.

This new system consisted of a mixture of 50% n-butanol, 45% water and 5% 0.05 M Phosphate buffer at pH 6.0. In this case the top phase was the stationary, non-polar one. The lower phase was mobile and polar. This type of phase system was used in the following experiment.

### Experiment 20

Serum which was obtained from a patient with cirrhosis of the liver was used. It was known to have a bilirubin total level of 8.3 mg. per cent with 4 mg. per cent of this direct diazo reacting.

1.5 ml. of this was deproteinized in the regular manner and the residue taken up in 2 ml. bottom polar phase.

The column itself was prepared by mixing 6 gm. kiesel-

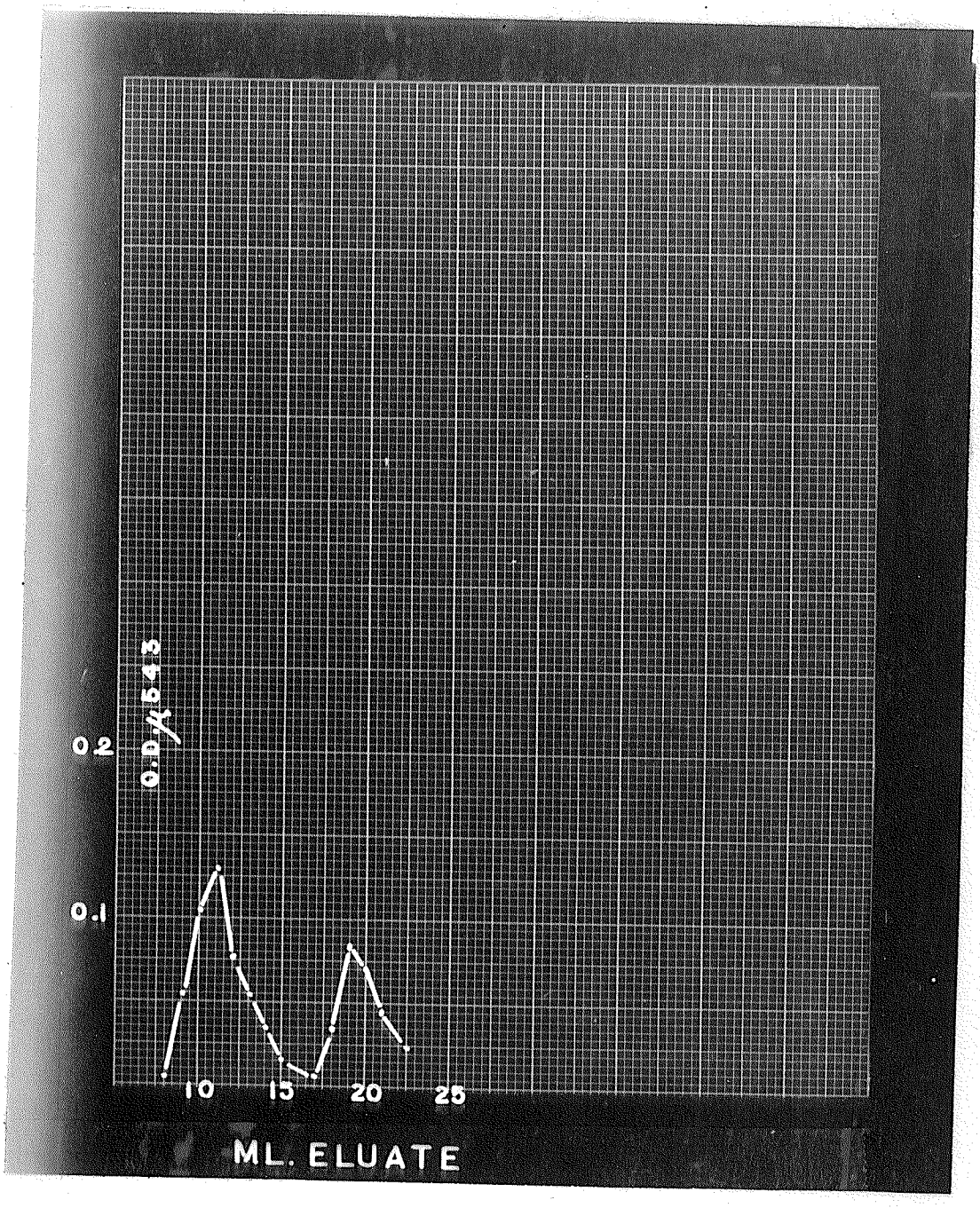


Figure 17

The Simultaneous Chromatographic Separation of Blood Serum from Cases of Obstructive Jaundice and of Hemolytic Jaundice

guhr with 3 ml. of top phase and then 17 ml. of bottom aqueous phase was added. The resulting slurry was poured into the column and packed with a stainless steel rod.

The serum residue in aqueous phase was then poured onto the column. More of this phase was added as needed to provide pressure and hasten the flow.

As the material moved down the column three bands of colour separated out. The first two bands of pigment moved down the column and slowly became separated from each other. The third band of pigment remained on the surface of the kieselguhr and did not move at all.

When sufficient separation of the pigments was achieved, the whole column was pushed out backwards with a glass rod and laid on a porcelain plate. The bands of pigment were cut out with a knife and washed out of the kieselguhr by shaking with more aqueous phase.

In this way three lots of pigment were obtained in aqueous phase. Some of this material from the two fast moving bands was examined in the spectrophotometer. These bands of pigment are called Pigment I and Pigment II by Billing.

Pigment I, the first coming off the column, showed no particular absorption maximum. There was a plateau between 4200 and 4500 A. Pigment II, however, showed a peak of absorption at 2700 A and a plateau and minor peak at 4500 A. The stationary band of pigment was not tested for absorption.

All three pigments were tested with diazo reagent after drying off the aqueous phase in vacuo at room temperature.

Pigment I and Pigment II were soluble in water and gave positive direct diazo reactions. The stationary pigment was only slightly soluble in water and only after addition of alcohol was a positive diazo reaction obtained.

It thus appears that Billing's work is confirmed. There are apparently two direct acting bilirubin pigments, as well as one indirect acting pigment.

## SECTION VI

Claireaux, Cole and Lathe described the characteristics of pigments which they had extracted from brain tissue from a case of kernicterus.

Waters (1955) described the production of bilirubin encephalopathy in newborn rats by the intra-peritoneal injection of solutions of bilirubin in alkali.

If it was possible to produce a kernicterus in laboratory animals, it seemed to be a useful source of bilirubin pigment for further study. The long standing argument as to whether the toxic agent in kernicterus was truly bilirubin or whether some other factor was responsible could possibly be studied in the newborn rat.

Day (1950) had shown that haemagglutination could not be held responsible. It was postulated that severe

clumping could result in lowered oxygen tension and consequent cell damage. The damaged cell could then take up bilirubin. This effect could not be demonstrated in experimental rats and furthermore Day cited the case of a child dying of kernicterus who showed no evidence of haemagglutination.

Day (1947) was able to produce icteric brain tissue in rats by first damaging the brain tissue with roentgen rays, and then tying off the common bile duct and by intravenous injection of bilirubin. He postulates that in naturally occurring kernicterus, the brain is first damaged by a toxin elaborated in the liver, or perhaps by antibody reaction. Following this the damaged cells are able to take up the bilirubin.

Billing, Cole and Lathe (1954) studied a series of newborn humans and were able to establish an inverse relationship between serum bilirubin levels and birth weight. Their theory is that the immature and small infant is not able to handle the excretion of bilirubin because of a specific defect in the liver. Other liver functions appear to be unaffected and the fault may be a very specific one which prevents the conversion of indirect bilirubin to the direct pigment. In utero the bilirubin is probably excreted across the placenta, but after birth the small infant does not have sufficient liver function to take care of it.

Najjar (1955) has made a study of liver slices to

determine the rate of speed at which they are able to concentrate bilirubin from serum. He found that it is an energy requiring process and that by using rat and rabbit liver slices it was possible to plot the uptake of bilirubin as a function of time, equilibrium being reached in 30 minutes at 37° C. Najjar believes that direct acting bilirubin is a ternary complex of bilirubin-metal-protein. Bilirubin-metal alone can exist at high pH but becomes dissociated at low pH. It can however be stabilized by linkage to protein. Electrophoresis studies have proved that this protein is albumen.

Vogel (1953) produced a yellow pigmented brain tissue in newborn kittens by injecting a strong solution of bilirubin directly into the brain tissue. On extracting this yellow tissue with chloroform Vogel found its absorption spectra to be the same as that of mesobilirubin. He supposes that bilirubin has somehow become reduced to mesobilirubin. Vogel was not able to demonstrate any damage in the neurons, although pigment was obviously imbedded in the cells. The disease was therefore not true kernicterus, since this always includes damage to the basal ganglia.

The present study was designed to try to show which of the two theories was correct, Vogel's or Claireaux's. The report of Waters previously mentioned leads one to believe that it should be relatively easy to produce staining of the newborn rat brain by the intra-peritoneal injection

of bilirubin. It was therefore decided to try to reproduce this effect by Waters Method.

Procedure--Eleven nests of newborn rats were used in the experiment. Within twenty-four hours of birth all were injected with one milligram of bilirubin for each gram of body weight. The solution of bilirubin was made up in 2 per cent sodium carbonate and titrated back to pH 7.5 with N HCL. In this way it was possible to have 0.5 ml. of fluid for intraperitoneal injection which contained 5 mg. Hoffman-LaRoche bilirubin. In this way the young rats received twice the dose of bilirubin that Waters was able to inject, because of the lower concentration of alkali.

In all, sixty-seven newborn rats were injected with bilirubin. Of these, twenty-four died, presumably as a result of the injections. Many of these were eaten by the mother rat and could not be examined. The survivors were killed with ether at various stages from two days old to about ten days old. Their craniums were then opened to expose the tissues to 10 per cent formalin and then to alcohol. After several days to harden the tissues the brains were removed and examined. On gross examination, and on coarse section, no evidence of the presence of bilirubin appeared. In a personal communication from Waters, he stated that the brain tissue staining was plainly visible to the naked eye.

Because it was thought possible that the formalin

or the alcohol had an adverse effect on bilirubin deposition, it was decided to examine some of the brains without fixing them first. This was done with one nest of rats, but again there was no apparent staining of the brain tissues.

Day (1947) had suggested that preliminary brain damage was necessary before the brain became kernicteric and therefore it was decided to try to damage the brains before the injections by making the rats anoxic. The newborn rats were divided into two groups, one group being placed in a tightly sealed glass jar and left long enough so that all showed marked signs of cyanosis. This cyanotic state was allowed to continue for about five minutes. After this the regular injections of bilirubin were given to the entire nest of rats. Three of these ten rats died and were eaten by the mother, but the other six were examined within four hours of death, using no fixatives on the brains. In the two groups of rats there was no apparent difference in those rats which had been rendered anoxic and those which had not. No bilirubin was found in any of them.

It was therefore concluded that it is not possible to produce bilirubin encephalopathy by simple intraperitoneal injection of a bilirubin solution in alkali.

## PART III

## CONCLUSIONS

The chromatographic method used for the study of bilirubin has elucidated some of its properties and has obscured others. The method itself as described by Cole and Lathe is not quantitative, but it is possible to obtain pigments in relatively pure form for further identification and study.

It has been shown that pure commercial bilirubin has apparently two different fractions which can be separated by chromatography.

The results of chromatography have lent weight to the arguments of those authors who claim that bilirubin is attached to protein. The finding of an absorption maximum at 2750 A for the direct acting fraction of bilirubin, leads one to believe that protein may be still attached to the bilirubin molecule even after deproteinization. Adding bilirubin to normal serum or to bovine albumen does not make any difference to the result of chromatographic separation.

It is possible to separate three distinct bilirubin fractions when all conditions of pH and temperature are favorable.

The difficulties attendant on getting the pigments into solution to be applied to the column led to the discovery

that the ability of bilirubin to dissolve in aqueous media is partly dependent on some substance to which it is attached. This substance is capable of being dissolved or removed by ether or chloroform.

It was found that direct acting bilirubin has a strong affinity for protein and adheres to it when deproteinization of serum is carried out with ammonium sulphate and alcohol. This bilirubin-protein combination somehow serves to protect direct acting bilirubin from ready oxidation on exposure to air.

All attempts to make bilirubin act as a fast moving pigment and to have it show the properties of a direct bilirubin have met with failure. The conclusion must be that indirect and direct bilirubin are separate entities.

It has been possible to demonstrate the presence of two direct acting bilirubin fractions which correspond to Pigment I and Pigment II described by Billing.

It has not been possible to demonstrate the existence of two indirect bilirubin fractions.

An attempt to produce kernicterus in newborn rats by intraperitoneal injection of bilirubin was not successful. It seems certain that the liver of the newborn rat is able to process very large quantities of injected bilirubin without harm to the animal. It is apparently necessary to cause damage to the brain cells of the newborn rats, before any bilirubin encephalopathy develops.

## SUMMARY

A study of the properties of bilirubin as it exists in blood serum has been made using a chromatographic technique developed by Howard and Martin (1950).

The limitations of the technique have been established and it has been found that although the method does not give quantitative results, it is sensitive enough to separate three distinct bilirubin pigments.

From the evidence of this study it seems likely that bilirubin in the direct acting form as well as the indirect form is attached to protein. The attachment of direct bilirubin to protein affords resistance to oxidation by exposure to air.

There is evidence of the existence of two distinct fractions of direct acting bilirubin which can be isolated from a strongly icteric blood serum.

An attempt has been made to produce bilirubin encephalopathy for the further study of the pathology of kernicterus, but this was entirely unsuccessful.

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