ELECTRO-CHROMOGRAMS OF HUMAN BILE

by

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INTRODUCTION

The clinical investigation of human bile samples has been poorly developed. In most hospitals, the routine chemical examination of bile is restricted to macroscopic and microscopic examination, determination of total bilirubin and eventually some other reactions. A more elaborate investigation is very time-consuming and possible only for research purposes[1]. We therefore tried to develop new means for rapid and more complete analysis of bile samples. This problem was the more attractive because of the great clinical interest *e.g.* in connection with bile concrement formation and with liver function research.

Paper electrophoresis provided a useful method for fractionating human bile constituents. It appeared possible to develop various staining methods by means of which a number of constituents could be visualised in the paper strips. Their mutual relationships and relative concentrations could be studied in single bile samples and conclusions drawn for physiological processes and pathological conditions. The essentials of the method were discussed in preliminary communications[2, 3].

MATERIAL AND METHODS

For the majority of the cases studied, fresh postoperative fistula bile samples were used. In several cases, bile samples were obtained by puncture of the hepatic duct, during operation, after clamping the cystic duct. In order to compare the 'liver-biles' with gall bladder bile samples, we used fresh samples, obtained by puncture of the gall bladder during abdominal operations for other than biliary diseases. Furthermore an examination was made of bile obtained from gall bladders, extirpated for stones or chronic inflammations*. A small number of bile-samples obtained at autopsies was used, but proved to be unsatisfactory. Finally the products of duodenal drainage were analysed and compared with the results of the analysis of pure bile samples.

Concentration of bile. Bile from the gall bladder had to be concentrated several times in most instances in order to obtain satisfactory diagrams. Samples from bile fistulae had to be concentrated 5 to 10 times; samples from duodenal drainage even

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References p. 48

further. Concentration was performed by placing fresh bile specimens of about 2 to 10 ml into small cellophane bags. The bags were placed in small beakers, partly filled with a 15% carboxymethylcellulose solution (Nyma) and put in the refrigerator for about 48 hours. Some of the bilirubin was lost but this proved to amount only up to 2% of the total bilirubin content of the sample. As soon as the concentration is estimated to be sufficient, the sample is used for paperelectrophoresis.

Paper-electrophoresis. From every bile sample, 3 to 5 diagrams are made by micro electrophoresis, using our modification of the apparatus of DE WAEL[4]. The essentials of the method were: filter-paper strips 4 × 24 cm, Whatman No. 1, suspended horizontally over two glass rods in a tray 36 × 44 × 17 cm, with their ends dipping into buffer-vessels containing barbiturate buffer 0.06 *M*, pH 8.6. Ten paper strips may be applied at a time. 0.03 ml of the bile is applied to each strip in the form of a thin band, 1 cm from the glass rod at the cathode side. An electric field of 7.5 V/cm is applied for 2 hours with the tray closed and eventually for another hour with the tray open in order to profit from the evaporation currents that may considerably sharpen the bands (electro-rheo-phoretic principle of MACHEBOEUF[5]). The cooling by evaporation is sufficient to keep the rise of the temperature in the strips under 3° C, at room temperature, even with the tray closed. After separation, the strips are dried at 110° C for 15 minutes.

Visualisation of various components. Two diagrams of each sample are split in two long strips. The total of 5 to 7 strips thus obtained are used for various staining methods.

a. Proteins

These were stained according to the method of TURBA[6] with azocarmine B (Bayer). The diagrams are placed for 15 minutes in a saturated solution of the dye in a mixture of methanol, acetic acid and water 5 : 1 : 4. The excess of dye is removed by bathing the diagrams 3 times for 5 minutes in a 10% acetic acid solution. This gives higher extinction of the dye remaining at the protein band, and a very low paper blank[4].

b. Bilirubin

One or more bands of bilirubinoids with a brown-yellow colour are visible. In strong acids these bands change to the bright green colour of biliverdin. Bathing the diagrams in fresh diazo reagent of Ehrlich does not give the red colour of the diazo-products. However, the diazo reaction in the paper strip may be obtained by thinly spraying the strip with the fresh reagent. The red colour then appears instantaneously. An excess of the reagent produces a bluish colour. This is prevented by using the following reagent freshly mixed: ethanol 96% 10 ml, sulfanilic acid 0.1% in water 10 ml, and sodium nitrite 0.5% in aqueous solution 0.6 ml. After spraying, the strips are dried in the air. The colour is relatively stable if the diagrams are kept in the dark.

c. Lipids

Lipids were stained by a modified procedure of SWAHN[7]. The diagrams are placed for 3 hours in a Sudan Black solution prepared as follows: 100 mg Sudan Black B.D.H. are suspended in 100 ml 60% ethanol. The mixture is warmed gently to boiling, filtered twice after cooling through a hard filter (Schleicher and Schüll No. 1575).

References p. 48

After staining, the strips are washed 3 times for 15 minutes in a 50% ethanol-water mixture. Lipids show blue-black bands, often of considerable intensity and on a practically white background. Some experience was necessary to control the staining process and especially the washing of the strips.

d. Bile acids

We adapted the reaction of ABE AND KAWAGUCHI[8] for paper strips. Free-hanging dry strips are sprayed with a 2% solution of vanillin in ethanol 96% until slightly wet. After complete drying in the air two pencil-marks are made, one at each end of the diagram. The strips are placed in Petri dishes, containing 85% phosphoric acid at a temperature of 70° C and put in an oven at 70° C for 4 minutes. Bile acids give a bright cherry-red colour. The bilirubin bands change to the bright green of biliverdin, giving a strong contrast. The paper slowly disintegrates in the strong acid after showing a remarkable contraction. This contraction is measured with the help of the pencil-marks. The position of the various bands is measured and the data corrected for the contraction. With coloured pencils a 'dummy' may be produced of the paper strip, for comparison with the other strips.

Fluorescent components

The native strips, placed at about 12 cm distance from an ultraviolet light source (Philips HP 120), show a variable number of fluorescent bands. The position, intensity, width and colour of the bands are noted and with the help of coloured pencils 'dummies' are produced for comparison with the other diagrams.

f. Urobilin

The strips are sprayed with the alcoholic zinc acetate reagent of SCHLESINGER. After drying, they are placed under ultra-violet light.

g. Cholesterol

Staining cholesterol on the paper strips with help of the reaction of Liebermann-Burchardt is possible but was omitted in favour an extraction method giving better quantitative results. The paper strips are cut into pieces 1 cm wide, extracted with chloroform and in these extracts the cholesterol is determined from the colour intensity of the reaction of Liebermann-Burchardt. Later on, cutting the diagrams in 3 equal pieces proved to be sufficient.

h. Alkaline phosphatase

The diagrams are cut into pieces 1 cm wide and their enzyme content estimated by the method of KING and ARMSTRONG.

RESULTS

The various strips are placed parallel and form together the 'electrochromogram' of a bile sample. Typical 'electrochromograms' of fistula bile and gall-bladder bile are given in Figs. 1 and 2. The location and relative position of the various bands may be studied. A semi-quantitative estimation of several compounds is easily possible. In this paper, only the results will be discussed of the diagrams of fistula bile and

References p. 48

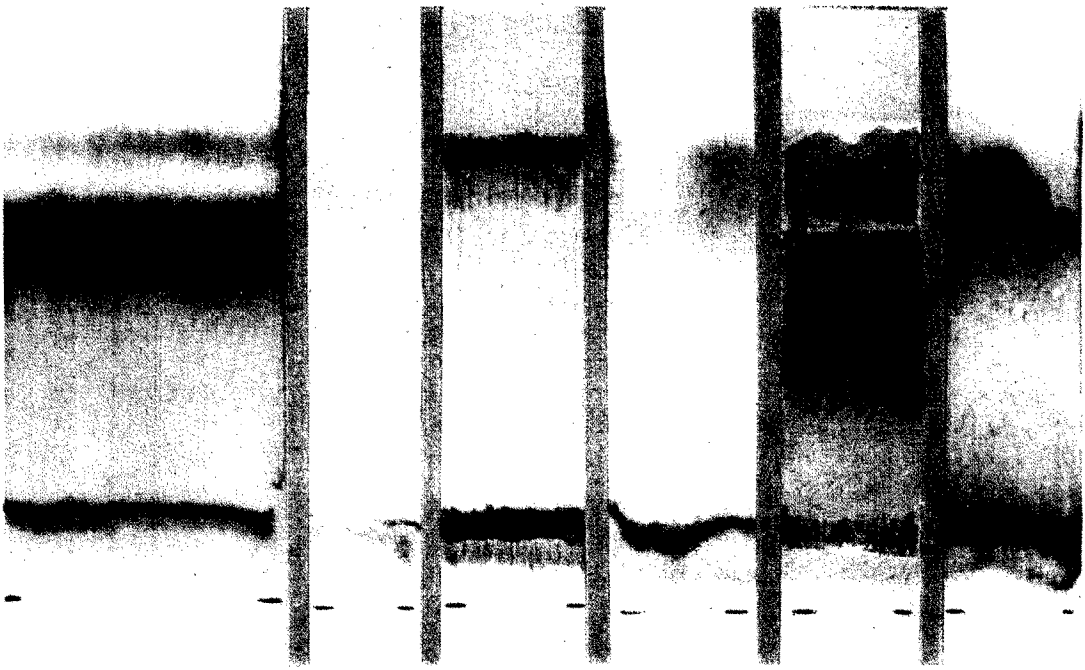

Fig. 1. Fistula bile.

1. Proteins
2. Lipids
3. Fluorescence
4. Unstained
5. Bile acids
6. Diazotation

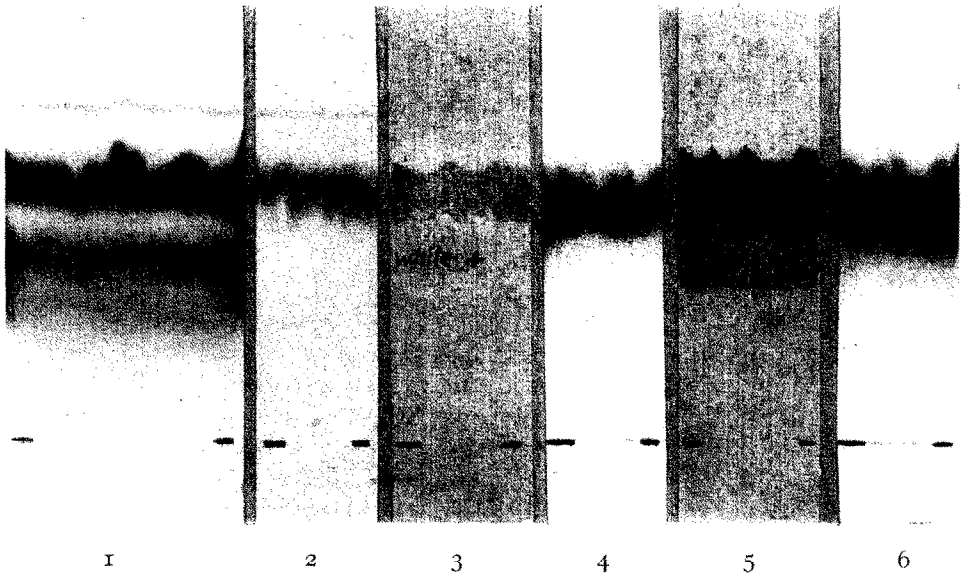

Fig. 2. Gall bladder bile.

1. Proteins
2. Lipids
3. Fluorescence
4. Unstained
5. Bile acids
6. Diazotation

gall-bladder bile. 106 samples of fistula bile from 27 patients were investigated, and 31 samples of gall-bladder bile. In 4 cases, bile from the hepatic duct could be secured during operations. In 2 more cases, both the gall-bladder bile and the bile from the hepatic duct were obtained simultaneously.

Proteins

Liver bile and gall-bladder bile have a much higher protein concentration than gastric juice or saliva. Four bands were distinguished with certainty, further called P (protein) 1,P2, P3 and P4. P1 is the fastest running protein, P4 remains at the starting point. This latter protein was encountered in only 4 cases of fistula bile. In bile samples from autopsies it was almost constantly present. In gall-bladder bile of cholecystitis patients, the protein concentration was always elevated and the P4 component always present. In the bile from normal gall bladders it was never found. P4 shows in the diagram a typical granulated appearance, which is also obtained with visible mucus from various sources. We therefore consider P4 as an abnormal bile constituent, consisting probably of visible mucus or related substances. In Figs. 1 and 2, no P4 is present.

P2 and P3 are the most constant protein fractions in fistula bile. P2 travels with a velocity, equal to or closely resembling that of serum albumin. It occurred in all samples analysed, almost always as the main protein fraction. P3 is found between P2 and the starting line as a diffuse band, suggesting that a mixture of proteins is present in this region. However, it must be kept in mind that in the complex medium of bile, substances may be present that have a disturbing influence upon the normal electrophoretic behaviour of proteins. This could clearly be seen when bile was added to serum. The picture of the serum proteins after electrophoresis was blurred. Analysis with electro-rheo-phoresis shows that P2 and P3 are two distinct proteins, as may be seen from Fig. 3.

P1, the fastest protein, was found in only 39 out of 106 samples of fistula bile. It was present as a very sharp and distinct band, in most cases in traces only. However in bile samples from normal gall bladders, this P1 component is the main protein (Fig. 2). Its high concentration cannot be the consequence of concentration in the gall bladder, because the P1 content is relatively much higher than that of the other visible components, as compared with fistula bile. Moreover it could not be detected in 67 of 106 samples of fistula bile. These facts suggest its excretion by the wall of the gall bladder. The identity of P1 is further disclosed by the lipid stain.

Lipids

With the lipid stain only one band could be distinguished. It is always found at the place of Protein 1. If no P1 was present, no lipid at its place could ever be detected. The lipid band always parallelled the extention and intensity of the P1 band. Therefore it is concluded that P1 is a *lipo*-protein. With ether, part of the lipids could be extracted but not all of them. Further physico-chemical data on this very interesting substance, which shows a remarkable tendency to complex-formation with bilirubin bile acids and chlolesterol (Fig. 2) will appear in a second communication. More evidence was collected that this lipoprotein is an excretion product of the gall bladder mucosa. Scrapings of the wall of well-washed fresh gall bladders showed considerable amounts of lipoprotein. Calculation of concentration ratios for gall bladder bile from

References p. 48

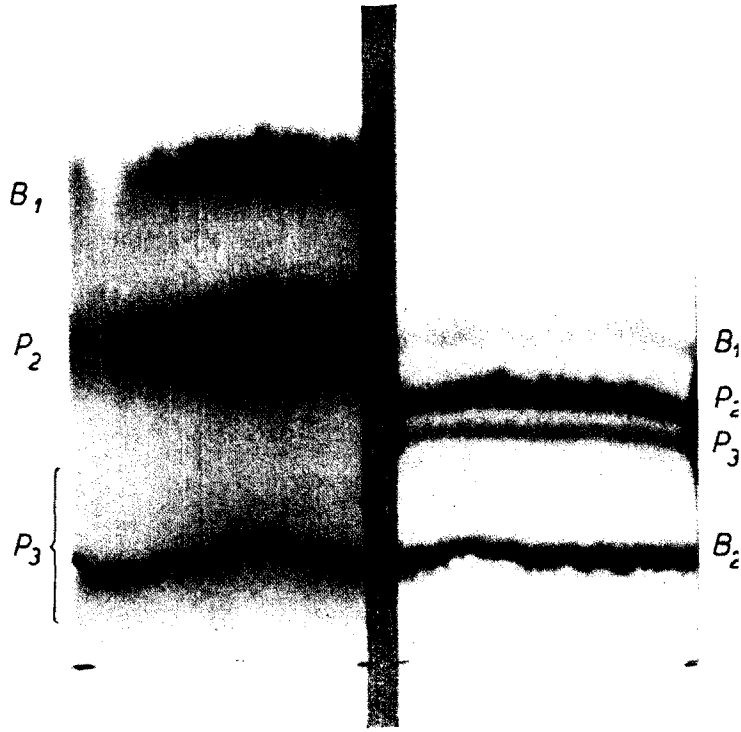

Fig. 3. Duplo of same bile sample as in Fig. 2.
1. Obtained with paper electrophoresis as described.
2. Obtained with electro-rheophoresis.
Proteins stained with azocarmin.

the data of POLONOVSKI AND BOURRILLON[1] showed concentration of bilirubin 4 times, cholesterol 3 times, proteins 2.5 times, but: lecithine 14 times, fatty acids 9 times, bile acids 11.5 times and choline 9.5 times, which would suggest either resorption of proteins, bilirubin and cholesterol by the gall bladder mucosa, or excretion of the fatty products. The latter possibility seems the more probable one in the light of our investigations.

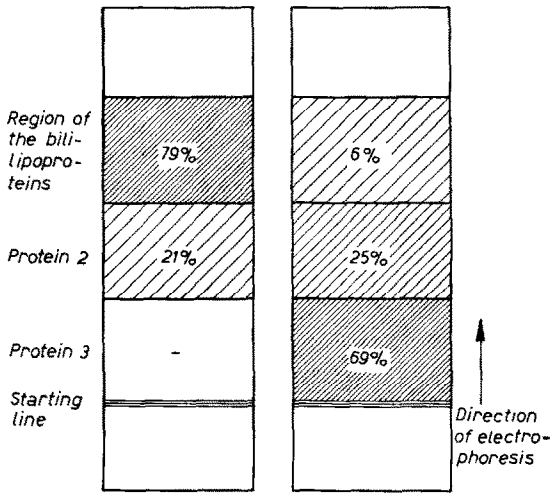

Fig. 4. Mean values for 8 bile samples from normal gall bladders and from 9 fistula bile samples.
Left: mean cholesterol contents on the paper strips of gall bladder bile.
Right: idem of fistula bile.

We called the lipoprotein of the gall bladder: 'bili-lipoprotein'. In this lipoprotein band, cholesterol could be detected by the reaction of Liebermann-Burchardt. A quantitative study of the binding of cholesterol to the 'bili-lipoprotein' was made. A number of diagrams from fistula bile and gall bladder bile samples were cut in 3

References p. 48

Fig. 5. 1. Fistula bile.
a. Proteins stained;
b. Diazotation; two pigment fractions visible.
2. Gall bladder bile.
a. Proteins stained;
b. Diazotation; all bile pigments bound to the lipoprotein.
3. Mixture of 1 and 2.
a. Proteins stained;
b. Diazotation; the slow-moving pigment fraction of the fistula bile has disappeared.

pieces: 1, corresponding with the area in which the bili-lipoprotein was found or could have been present; 2, the mid-piece of the diagram, corresponding to the place of P2; 3, the remaining area, between 2 and the starting line. The cholesterol contents of these pieces were determined in their chloroform extracts with the Liebermann-Burchardt reaction and the extinctions measured at 660 mμ with the Beckman spectrophotometer. The relative quantities in the various regions were calculated in percents of total cholesterol content of each strip. Mean values for 8 bile samples from normal gall bladders and from 9 fistula bile samples are given in Fig. 4.

From this figure it is apparent that in gall bladder bile the cholesterol is largely bound to the bili-lipoprotein. In fistula bile however, no cholesterol was found in this region if no bili-lipoprotein was detectable. This strongly suggests complex binding of cholesterol in the gall bladder to the bili-lipoprotein.

Bile pigments

One of the most striking phenomena in the diagrams of a number of fistula bile samples is the appearance of two distinct bilirubinoid bands, further called B1 and B2. B1, the faster moving and more polar pigment is more easily soluble in several organic solvents. If filter paper strips are placed at pH 6.8 in a 3 % solution of human albumin, both pigments are slowly dissolved. With the diazo-reagent, they *both* give a direct van den Bergh reaction. An increasing amount of evidence for the existence of two different kinds of direct reacting 'bilirubin' has accumulated in recent years[1, 9, 10]. The identity of our two direct reacting bile pigments with those of COLE, LATHE AND BILLING[10] remains to be proven.

B1 was present in all samples of fistula bile. In practically all the cases it was the main component. This fraction showed always a more diffuse band than the B2 fraction. In fistula bile samples, both bilirubin fractions travel with their own velocities and no relation with protein is found. The bile acids also do not occupy the same area as the bile pigments (Fig. 1). B2 was present in 87 out of 106 fistula bile samples. It has never been found in normal gall-bladder bile. A remarkable fact is that B2 was absent in those samples of fistula bile that contained appreciable amounts of bili-lipoprotein. There exists a reverse relation between the presence of the bili-lipoprotein and the slow moving bile pigment. B2 bands were remarkably sharp. Evaporation and adsorption, together with the electrophoretic forces, probably play a role in the formation of the band. The picture of gall-bladder bile again is very different. One band of bile pigment is found, running with approximately the same velocity as B1. It is always found exactly at the position of the bili-lipoprotein and follows the irregularities that may appear in the bili-lipoprotein band. The bile pigments seem to be attached to the lipoprotein. The fact that pigment B2 has never been found when lipoprotein was present in appreciable amount would suggest that this pigment too is bound by adsorption to the lipoprotein. In order to investigate this further, a typical sample of fistula bile, containing both the bile pigments, was mixed with a typical sample of gall-bladder bile, with a high concentration of bili-lipoprotein. In this mixture pigment 2 disappeared by adsorption to the bili-lipoprotein (Fig. 5).

Bile acids

In fistula bile they are found between the two bilirubin fractions. Overlapping never occurs. They are present in strongly variable concentrations. In gall-bladder

References p. 48

bile, however, they are largely found at the position of the lipoprotein band. Here, a complex lipoprotein-bilirubin-bile acid seems to exist.

Fluorescence diagrams

A variety of bands is found, some connected with already known components, other still of unknown origin.

The lipoprotein band shows a bright yellow fluorescence; the two bilirubin bands show a pinkish-brown fluorescence.

White-yellow bands of variable intensity are often present between B1 and B2. In several diagrams small red bands were seen, possibly given by porphyrins. The investigations in this direction are being continued.

SUMMARY

Fivefold diagrams of concentrated samples of human bile were obtained with paper electrophoresis. In these diagrams were distinguished by means of various staining methods: proteins, lipids, bile pigments, bile acids and cholesterol. The series of diagrams from each bile sample thus obtained is called an 'electro-chromogram'. These electro-chromograms permit the study of various important bile constituents and their interrelationships. 'Electro-chromograms' were made of 112 bile samples from fistulae or obtained by puncture of the hepatic duct during surgery, and 31 bile samples from gall-bladders obtained in surgery.

It appeared that:

1. Liver bile is not only quantitatively but also qualitatively different from the concentrated bile in the gall-bladder. Bile of the 'gall-bladder-type' contains a high concentration of a lipoprotein complex, probably excreted by the mucosa of the gall-bladder. To this lipoprotein, bilirubins, cholesterol and bile acids are complexly bound. The lipoprotein seems to play a major rôle as a stabilizing factor of the solution in the gall-bladder. It is called 'bili-lipoprotein'. Its role in cholelithiasis is the subject of further investigations.

2. Bile of the 'liver type' contains no bili-lipoprotein or in some cases only a small quantity. Two other proteins are always present. Often, two different, direct-reacting bilirubin fractions are found. Bilirubins and bile acids occupy different areas on the filter-paper strips. No evidence of binding between proteins and bilirubin, or of bile acids to bilirubin has been found.

3. A number of still unindentified substances is present.

RÉSUMÉ

Les auteurs ont obtenu par électrophorèse sur papier des diagrammes quintuples de prises concentrées de bile humaine. Dans ces diagrammes les constituants suivants sont rendus visibles par différentes méthodes de teinture: protéines, lipoïdes, pigments biliaires, acides biliaires et cholestérol. La série de diagrammes ainsi obtenue à partir de chaque prise de bile s'appelle 'électro-chromogramme'. Ces électro-chromogrammes permettent l'étude de différents constituents importants de la bile et de leurs relations mutuelles. Les auteurs ont fait des électro-chromogrammes de 112 prises de bile de

References p. 48

fistules ou obtenues par ponction du canal hépatique au cours d'une opération, et de 31 prises obtenues à partir de la vésicule biliaire au cours d'une opération.

Voici les résultats obtenus:

1. La 'bile de foie' est, non seulement quantitativement mais aussi qualitativement, différente de la bile concentrée qui se trouve dans la vésicule biliaire. La bile du type 'vésicule biliaire' contient, en concentration élevée, un complexe de lipoprotéine, qui est probablement sécrété par la muqueuse de la vésicule biliaire. Les bilirubines, le cholestérol et les acides biliaires sont liés à cette lipoprotéine d'une manière complexe. La lipoprotéine semble jouer un rôle important comme facteur stabilisant de la solution contenue dans la vesicule biliaire. Elle s'appelle 'bili-lipoprotéine'. Son rôle dans la cholélithiase formera l'objet d'investigations ultérieures.

2. La bile 'du type foie' ne contient pas ou, en certains cas, une petite quantité de bili-lipoprotéine. Deux autres protéines sont toujours présentes. L'on trouve souvent deux fractions de bilirubine réagissant directement. Les bilirubines et les acides biliaires occupent sur les bandes de papier filtre des aires différentes. Aucune preuve d'une liaison entre les protéines et la bilirubine et entre les acides biliaires et la bilirubine n'a été trouvée.

3. Un certain nombre de substances non encore identifiées est présent.

ZUSAMMENFASSUNG

Fünffache Diagramme von konzentrierten Proben menschlicher Galle wurden papierelektrophoretisch erhalten. In diesen Diagrammen wurden mit Hilfe verschiedener Färbungsmethoden Proteine, Lipoïde, Gallenpigmente, Gallensäuren und Cholesterol sichtbar gemacht. Die so für ein und dieselbe Gallenprobe erhaltene Reihe von Diagrammen, heißt 'Elektrochromogramm'. Sie gestatten das Studium verschiedener wichtiger Gallenbestandteile und deren gegenseitige Abhängigkeit. Elektrochromogramme wurden von 112 Gallenproben aufgenommen, welche entweder aus Fisteln oder durch Punktion des Leberganges bei Operation erhalten worden waren, außerdem von 31 Gallenproben welche während der Operation aus Gallblasen entnommen waren. Die folgenden Ergebnisse wurden erhalten:

1. 'Lebergalle' ist nicht nur quantitativ sondern auch qualitativ verschieden von der konzentrierten Galle in der Gallenblase. Galle vom 'Gallblasentypus' enthält in hoher Konzentration einen Lipoproteinkomplex welcher wahrscheinlich von den Schleimhäuten der Gallenblase abgesondert wird. An dieses Lipoprotein sind die Bilirubine, Cholesterol und die Gallensäuren komplex gebunden. Das Lipoprotein scheint als stabilisierender Faktor der Lösung in der Gallenblase eine wichtige Rolle zu spielen. Es wird 'Bili-Lipoprotein' genannt. Seine Rolle bei der Cholelithiasis wird weiter untersucht.

2. Galle vom 'Lebertypus' enthält kein Bili-Lipoprotein oder in einigen Fällen nur eine kleine Menge davon. Zwei andere Proteine sind immer zugegen. Öfters werden zwei verschiedene direkt reagierende Bilirubinfraktionen gefunden. Bilirubine und Gallensäuren nehmen auf den Filtrierpapierstreifen verschiedene Flechen ein. Es wurde kein Hinweis auf eine Bindung zwischen Proteinen und Bilirubin und von Gallensäuren und Bilirubin gefunden.

3. Eine Anzahl von noch nicht identifizierten Substanzen ist gegenwärtig.

References p. 48

РЕЗЮМЕ

Пятикратные диаграммы концентрированных проб человеческой желчи были иолучены с помощью электрофореэа на бумаге. В этих диаграммах при помощи различых окрашивающих методов были ясно видимы: белки, липиды, желчные пигменты, желчные кислоты и холестероль. Серии диаграмм от каждой желчной пробы таким образом полученные были названы „электро-хромограммами". Они дают возможность изучать различные важные желчные компоненты и их взаимные связи. Электрохромограммы были сдсланы от 112 проб желчи из фистулы или получены иутем прокола печеночного протока во время операции, и 31 проба желчн из желчного пузыря полученная во время исследований.

Оказалось, что:

1. желчь печени не только количественно, но и качественно отличается от концентрированной желчи в желчном пузыре. Желчь „типа желчного пузыря" содержит высокую концентрацию липопротеиннаго комплекса, вероятно, выделяемого слизистой оболочкой желчного пузыря. С этим липопротеином комплексно связаны билирубины, холестороль и желчные кислоты. Липопротеин повидимому играет более важную роль как стабилизирующий фактор в растворении в желчном пузыре. Он назван „билилипипротеином". Его роль в холелитиазисе предмет дальнейших исследований.
2. Желчь „печеночного типа" не содержит били-липопротеина или в некоторых случаях только в небольшом количестве. Два других протеина всегда находятся. Часто встречаются два различные вызывающие прямую реакцию фракции билирубина. Билирубин и желчные кислоты занимают различные площади на полосах фильтровой бумаги. Не найдена связь ни между протеинами и билирубином, ни между желчыми кислотами и билирубином.
3. Были обнаружены еще неопознанные вещества.

REFERENCES

1 M. POLONOVSKI AND R. BOURRILLON, *Bull. soc. chim. biol.*, *34* (1952) 703.
2 J. C. M. VERSCHURE, Commun. on Sep. 25, 1954 before the medical section of the Thymgenootschap (*Ann. Thymgenootschap*, *43* (1955) I).
3 J. C. M. VERSCHURE, *3rd Intern. Congr. Biochem., Brussels, 1955*, communication 16 - 8.
4 J. C. M. VERSCHURE AND F. M. C. HOEFSMIT, *Ned. Tijdschr. Geneesk.*, *98* (1954) 3410.
5 M. MACHEBOEUF, *Chem. Weekblad*, *49* (1953) 237.
6 F. TURBA AND A. J. ENEKEL, *Naturwiss.*, *37* (1950) 93.
7 B. SWAHN, *Scand. J. Lab. Clin. Med.*, *5* (1953) suppl. 9.
8 SH. KAWAGUCHI, *J. Biochem.*, *28* (1938) 445.
9 N. H. MARTIN, *J. Am. Chem. Soc.*, *71* (1949) 1230.
10 P. G. COLE, G. H. LATHE AND B. H. BILLING, *Biochem. J.*, *57* (1954) 514.

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