Auf Grund der Untersuchungen kann festgestellt werden, dass im Gegensatz zur Schweinegalle Menschen- und Ochsengalle in bezug auf die Gallensäurezusammensetzung als physiologisch verwandte biologische Stoffe zu betrachten sind.

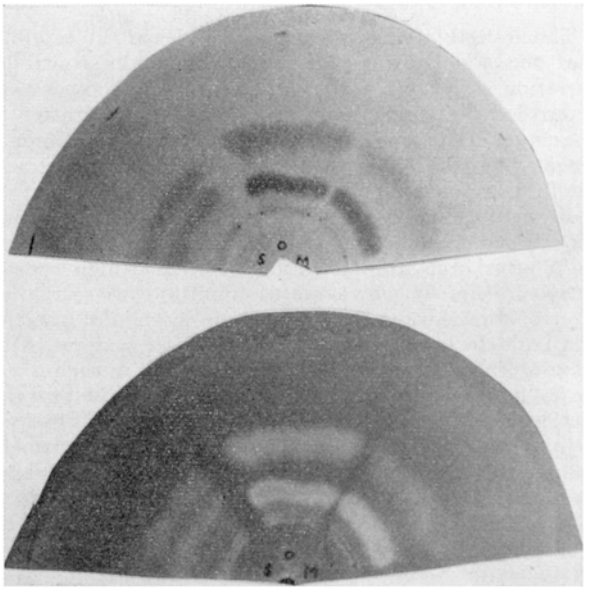

Abb. 3. Aufnahme im Tages- und UV-Licht. Rundfiltertechnik
S Schweinegalle;
O Ochsengalle;
M Menschengalle.

Beschreibung der Versuche

Trennung und Identifizierung der Gallensäuren:

Papier: Lufttrockene Schleicher & Schüll 2043b Mgl-Papierstreifen der Grösse 8 × 30 cm oder Rundfilter.

Das Papier wird genau 8 min mit 20%iger 1,2-Propylenglykol-Lösung in Chloroform imprägniert, dann zwischen Filterpapierbogen abgepresst und 1–2 min an der Luft trocknen gelassen.

Die Gallensäuren und Gallenhydrolysate werden aus 1–2%iger Methanol-Chloroform(1:1)-Lösung aufgetragen und die Chromatogramme nach der aufsteigenden Methode bzw. Rundfiltertechnik mit dem Lösungsmittelsystem Xylol-Methyläthylketon 1:1 entwickelt.

Entwicklungszeit: 2–3 h. Versuchstemperatur: 22°C ± 2°.

Das Imprägnierungsmittel lässt sich etwa eine Woche lang gut verwenden. Es ist zweckmässig, das Lösungsmittelsystem nach 2–3 Entwicklungen frisch vorzubereiten.

Nach Beendigung der Entwicklung werden die Chromatogramme im Trockenschrank mit Umluft $^1/_2$ h bei 100 bis 110°C vollständig von Lösungsmittel und Propylenglykolimprägnierung befreit.

Zur Sichtbarmachung der Flecken dient 20%ige Antimontrichloridlösung in Chloroform, durch welche die getrockneten Chromatogramme gezogen werden. Nach anschliessender Erwärmung, 5–10 min auf 100–110°C, erscheinen die Flecken mit rotvioletter Farbe. Die Auswertung wird im filtrierten UV-Licht vorgenommen (vgl. Tabelle 1).

Empfindlichkeit der Methode: 5–10 µg.

Für die Sichtbarmachung kann auch 30%ige wässerige Phosphorsäurelösung als Sprühreagens benutzt werden. Nach Trocknen auf 110–120°C erscheinen die Flecken im UV-Licht mit blauvioletter Fluoreszenz.

Hydrolyse der Gallensäfte:

50–100 ml der zu hydrolysierenden Galle werden mit der entsprechenden Menge Natronlauge auf etwa 10% an Natriumhydroxyd eingestellt und am Rückflusskühler 18–24 h gekocht. Zur Beseitigung der Schaumneigung bewährte sich ein Zusatz von 0,1% Bayer-Entschäumer E 100 in alkoholischer Lösung.

Nach Beendigung der Hydrolyse wird das alkalische Reaktionsgemisch mit 20%iger Schwefelsäure zur Fällung der freien Gallensäuren angesäuert (pH ~ 3–4).

Die ausgefallene braune amorphe Substanz wird mit Wasser stark ausgewaschen und im Trockenschrank getrocknet. Nach mehrmaligem Umkristallisieren aus Methanol-Chloroform (1:1) lässt sich das Gemisch der freien Gallensäuren in schöner kristalliner Form erhalten.

Summary

In cases of bile-secretion disturbances substitution-therapy can be performed using suitable animal biles equivalent to human bile. Comparative investigations have been made to establish which animal bile is in practice the most suitable for bile substitutiontherapy based on its bile acid components.

A new paper partition chromatographic method has been applied for separation and identification of free bile acids. Ascending development was used on Schleicher & Schüll 2043b Mgl paper impregnated with 20 v/v% propylene glycol in chloroform. The xylene-methylethylketone 1:1 solvent system gave good separation. The bile acids can be detected by immersing the chromatograms in 20 w/v% $SbCl_3$ in chloroform followed by drying and heating for 5–10 min at 100–110°C. The spots show intense reddish-violet or blue fluorescence in filtered UV-light (see Table 1).

These investigations have shown that human and ox biles are, in contrast to pig bile, physiologically related biological substances regarding their bile acid components.

STUDIORUM PROGRESSUS

Identification of Human Serum Proteins Binding Iron, Copper, and Thyroid Hormones by Starch Gel Electrophoresis

By A. C. Allison*

It has been recognized for many years that metal ions and thyroid hormones in the blood stream are bound to plasma proteins. The high resolution achieved by the starch gel electrophoresis technique of Smithies[1], and the use of the discontinuous buffer system of Poulik[2], has permitted the accurate identification of the serum proteins responsible for the binding and has given some new information about their genetical control and properties. A typical starch gel pattern of human serum proteins

* National Institute for Medical Research, London.

[1] O. Smithies, Biochem. J. *61*, 629 (1955).

[2] M. D. Poulik, Nature *180*, 1477 (1957).

is shown in Figure 1. It is widely believed that the high resolving power of the starch gel system lies in a molecular sieving effect of the supporting medium, which allows greater mobility of small molecules than large molecules[3]. Hence proteins are separated on the basis of differences in size as well as charge. Consistent with this interpretation is the low mobility in starch gels of high-molecular weight components such as β-lipoprotein[3,4], 19 S α_2 glycoprotein[3], macroglobulin[5], and thyroglobulin (see below).

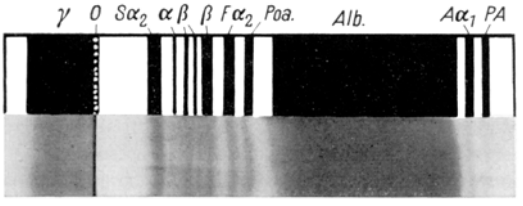

Fig. 1. – Starch gel electrophoresis of serum proteins in borate buffer, showing the main components, from left to right: γ globulin; origin; slow α_2 globulin; $\alpha\beta$ components (haptoglobins); β (transferrin C); fast α_2 (including caeruloplasmin); post-albumin; albumin; acidic α_1-glycoprotein[5a]; prealbumin.

Iron. – It has been established that iron is carried in plasma in the form of a ferric complex with a β-globulin of molecular weight about 88,000 which has been called 'transferrin' or 'siderophilin'[6]. Two-dimensional electrophoresis shows that the β-globulins are resolved into two main components in starch gels[3]. One component has a low mobility in the gel, and is easily identified as β-lipoprotein[3,4]. The other is a β-globulin band which in some sera shows splitting into two approximately equal components[7]. The splitting occurs only in certain families and

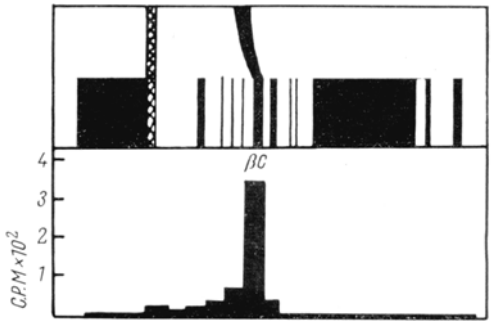

Fig. 2. – Above, migration of purified transferrin and normal serum on starch gel electrophoresis. The transferrin band is continuous with the βC component. Below, radioactivity in different serum fractions after ^{59}Fe labelling. The peak of activity corresponds with the βC component.

[3] M. D. POULIK and O. SMITHIES, Biochem. J. *68*, 636 (1958).

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[5a] K. SCHMID, J. Amer. chem. Soc. *72*, 2816 (1950). – H. E. WEIMER, J. W. MEHL, and R. J. WINZLER, J. biol. Chem. *185*, 561 (1950).

[6] C. B. LAURELL, and B. INGELMAN, Acta chem. scand. *1*, 770 (1947). – J. L. ONCLEY, G. SCATCHARD, and A. BROWN, J. Phys. coll. Chem. *51*, 184 (1947). – H. E. SCHADE, R. W. REINHART, and H. LEVY, Arch. Biochem. *2*, 170 (1949). – E. J. COHN, Ann. int. Med. *26*, 341 (1947). – H. E. SCHULTZE, K. HEIDE, and H. MULLER, Behringwerk-Mitt. No. 32, 24 (1957).

[7] O. SMITHIES, Nature *180*, 1482 (1957). – W. R. HORSFALL and O. SMITHIES, Science *128*, 35 (1958). – O. SMITHIES, Nature *181*, 1203 (1958). – H. HARRIS, E. B. ROBSON, and M. SINISCALCO, Nature *182*, 452 (1958).

appears to be under the control of a single pair of genes. The majority of human subjects in all populations so far tested show only a single β band, a phenotype which has been termed βC. Other phenotypes described include β B_1 C, β B_2 C, β C D_2 and β C D_1. The B components have a higher and the D components a lower anodal mobility than C at pH 8·4.

The investigations which will be described establish that the β-globulin which shows genetically controlled variation is, in fact, transferrin. In the first experiment a purified preparation of transferrin was submitted to starch gel electrophoresis together with a normal human serum. The transferrin band was clearly continuous with the βC component (Fig. 2). In the second experiment the position of radioactive iron carried by serum proteins was determined. Serum was obtained from 3 human subjects 24 h after oral administration of 10 μC high specific activity ^{59}Fe. The sera were submitted to starch gel electrophoresis using the starch grain method of insertion and both the borate and *tris*-borate buffer systems. After staining the gel was cut into equal sections in such a way as to have the main protein components in separate sections, and the radioactivity in each section was assayed in a well-type scintillation counter. A correction for volume was made by counting sections of a gel containing a standard iron solution. A typical result is shown in Figure 2. The peak of activity clearly coincides with the β band; the slight activity between this band and the origin could be due to some trailing of this component on the upper and lower surfaces of the gel. Similar results were obtained when serum was incubated with 0·4 μg $^{59}Fe^{3+}$ per ml and dialysed against buffer before electrophoresis. The latent iron-binding capacity in the two sera used was 0·5 and 0·6 μg per ml. Two African sera showing double β-globulin bands, corresponding to the phenotypes β C D_1 and β C D_2, were incubated with $^{59}Fe^{3+}$ in concentrations of 0·4 μg per ml, this again being less than the latent iron-binding capacity of the sera. After dialysis the sera were submitted to prolonged electrophoresis in the discontinuous buffer system, which ensures good separation of the β-globulin components, in confirmation of the report of HARRIS *et al.*[7]. The result is shown in Figure 3B; most of the radioactivity was distributed between the two β peaks.

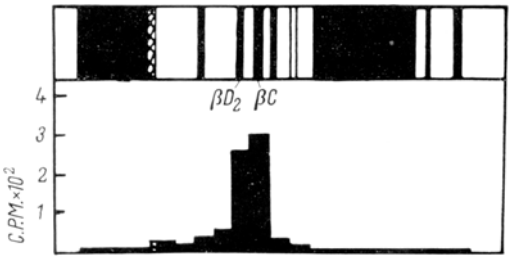

Fig. 3. – Radioactivity after starch gel electrophoresis in *tris*-borate buffer of a T_f^C/T_f^{D2} serum incubated with ^{59}Fe. The radioactivity is nearly equally distributed between the βC and βD_2 peaks.

Further observations were made by immunoelectrophoresis, which has already been adapted to the starch gel system[3,8]. After preliminary electrophoresis in *tris*-borate buffer[2], a thin slice of starch gel was stained to show the β components. A narrow longitudinal section of the remainder of the gel was placed in a large Petri dish

[8] M. D. POULIK, Nature *177*, 982 (1956).

and just covered with molten 1·5% agar at 40°C. When the agar had set, a trough was cut parallel to the starch gel and filled with a rabbit antiserum to human transferrin. In β-C sera a single precipitation line in the position of the β component was observed, but in βCD_1 and βCD_2 sera two lines, continuous at one point, were apparent (Fig. 4). These experiments show that both βC and βD components react with anti-transferrin serum.

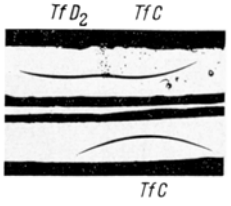

Fig. 4. – Micro-immunoelectrophoresis, using *tris*-borate buffer, of sera from Tf^C/Tf^{D_2} (above) and Tf^C/Tf^C below.

SMITHIES[9] has independently concluded that the β-globulins subject to genetical variation are transferrins. He has made the appropriate suggestion that they should be known as transferrin or *Tf* types C, B_1C, B_2C, CD_1, and CD_2, the corresponding genes being Tf^C, Tf^{B_1}, and so forth. Incidentally, the finding of HORSFALL and SMITHIES[7] that the normal β-band is lacking in starch gel patterns of a Tf^B/Tf^B subject suggests that the β-band consists largely of transferrin. The observations presented in this paper indicate that the β variants can carry iron and react with anti-transferrin serum. They may differ from the normal transferrin molecule only in small details of structure and charge, in the same way that the abnormal hemoglobin types S and C appear to differ from the normal adult type only in single amino-acid substitutions in each half-molecule[10].

Drs. D. R. BANGHAM and D. E. H. TEE kindly allow me to quote their finding that a very similar β-globulin variation occurs among monkeys (*Macaca mulatta*), some animals having a single and others a double component. Whether any of the complicated genetically-controlled β-globulin variations in other species[11] concern transferrins is at present unknown.

Copper. – The copper binding protein, caeruloplasmin, has aroused interest recently because it is much reduced in amount in, or altogether absent from, the sera of subjects with WILSONS disease[12]. Failure of synthesis of this protein may indeed be the primary defect in subjects homozygous for the abnormal gene. Heterozygous carriers of the gene frequently have subnormal caeruloplasmin levels. Caeruloplasmin has been characterized as an α_2-globulin of molecular weight about 150,000[13]. DE GROUCHY[14] using the *p*-phenylenediamine staining method of URIEL[15] states that after starch-gel electrophoresis the caeruloplasmin activity is found in the fast α_2 region. This has been confirmed by experiments in which purified caeruloplasmin has been run alongside serum in a starch gel. The caeruloplasmin band was clearly continuous with the fast α_2 component and both were stained by *p*-phenylenediamine.

Thyroxine and other iodine-containing compounds. – It is generally accepted that thyroxine is normally bound to a plasma protein having an electrophoretic mobility on filter paper at pH 8·6 between those of α_1- and α_2-globulins[16]. However, reports have appeared of a thyroxine-binding protein in the prealbumin region in sera of patients with nephrosis and in cerebrospinal fluid[17]. In a preliminary communication[18] it has been stated that after starch gel electrophoresis of serum proteins thyroxine was located mainly in the prealbumin region. Moreover, when a prealbumin-rich fraction was added to normal human serum[19], some of the thyroxine label was found in the prealbumin region, from which it was concluded that prealbumin and the α-component are two distinct thyroxine-binding proteins, albumin being a secondary binding protein effective only at higher concentrations of thyroxine. TATA[20] has presented evidence in support of an alternative hypothesis, that the thyroxine-binding property of the α-globulin fraction is due to prealbumin present in this region after paper electrophoresis in the form of a complex with another serum protein. Purified prealbumin migrates in the typical position ahead of albumin on filter paper electrophoresis at pH 8·6.

Fig. 5. – Pattern obtained by starch gel electrophoresis of purified caeruloplasmin and normal human serum. The caeruloplasmin band is continuous with the fast α_2 band.

During the course of the present study, the position of ^{131}I-radioactivity was determined after starch gel electrophoresis of sera to which labelled thyroxine and triiodothyronine had been added, and of subjects given tracer and therapeutic doses of radioiodide. The first experiments were carried out with serum from normal adult subjects to which, after preliminary dialysis against polyvinylpyrrolidine to remove as much thyroxine as possible, ^{131}I-L-thyroxine and ^{131}I-3,5,3′-triiodo-L-thyronine were added in concentrations of 0·1 to 0·6 μg per ml. After electrophoresis in borate buffer and staining of the gels they were cut into sections the radioactivity of which was assayed in a well-type scintillation counter. A typical result is shown in Figure 6. It is clear that when thyroxine or triiodothyronine in concentrations up to 0·2 μg per ml is added to serum, nearly all the radioactivity is concentrated in the faster-moving of the two prealbumin bands. When 0·4 μg per ml of these compounds was added, the radioactivity was about equally distributed between the first prealbumin band and albumin. These results suggest that prealbumin can bind about 0·2 μg thyroxine or triiodothyronine per ml serum, which is less than that reported by RICH and BEARN[18] but approximately the same as the α-thyroxine-binding-protein is reported to bind on filter paper electrophoresis in barbiturate buffer[16].

[9] O. SMITHIES, personal communication.

[10] V. M. INGRAM, Nature *180*, 326 (1957). – J. A. HUNT and V. M. INGRAM, Nature *181*, 1062 (1958).

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[16] J. ROBBINS and J. E. RALL, Rec. Adv. Hormone Res. *13*, 161 (1957).

[17] J. ROBBINS, J. E. RALL, and M. L. PETERMAN, J. clin. Invest. *36*, 1333 (1957).

[18] C. RICH and A. G. BEARN, Endocrinology *62*, 687 (1958).

[19] S. H. INGBAR, Endocrinology *63*, 256 (1958).

[20] J. TATA, Nature *183*, 877 (1959).

These results focus attention upon prealbumin as the main thyroxine-binding protein of human serum. Prealbumin has been purified and its properties studied by Schultze *et al.*[21]. We have run their preparation alongside normal human serum in starch gels and found it to be continuous with the first prealbumin band. This protein is quite different from the acidic α_1 glycoprotein which Rich and Bearn[18] suggest may be the thyroxine-binding protein, but which, in fact, appears to be continuous with the second prealbumin band and does not bind thyroxine. Prealbumin has a molecular weight of 61,000 and is characterized by a tryptophan content (2·5%) which is much higher than that of other serum proteins. Prealbumin has an intense tryptophan fine-structure absorption band in the ultraviolet at 2901 A and another fine-structure band at 2839 A[22]. A number of investigations[21] have established that there is a high content of prealbumin in cerebrospinal fluid, where, as already stated[16,17], it binds thyroxine.

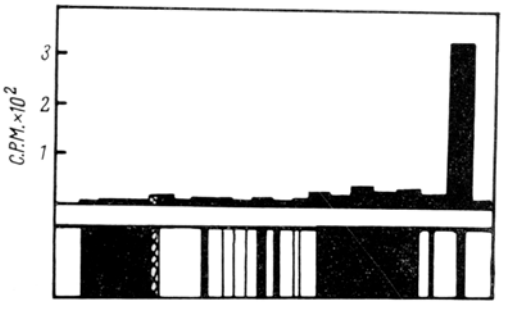

Fig. 6. – Radioactivity in various protein bands of normal human serum incubated with 0·2 µg. ^{131}I-thyroxine per ml and submitted to starch gel electrophoresis.

A thyroxine-binding protein with many of the properties of prealbumin has also been found in human and monkey amniotic liquor[23]. This protein reacts with antisera against prealbumin[20], moves ahead of albumin on starch gel electrophoresis and has a high tryptophan content. These observations suggest that prealbumin may cross the placenta, carrying maternal thyroxine. Little is known about the passage of thyroxine across the placenta of primates, but in rabbits significant proportions of labelled thyroxine injected into the mother appear in the fetal circulation only after about the 30th day of gestation[24]. At about the same time a thyroxine-binding protein appears in fetal plasma having the same electrophoretic mobility as the corresponding maternal protein; but whether it is of fetal or maternal origin has not been established.

Some observations have also been made on sera of patients with carcinoma of the thyroid gland treated with radioiodide. In the majority of cases, three well-defined peaks of activity were observed after atarch gel electrophoresis (Fig. 7). One peak corresponded to the first prealbumin band, and probably represents thyroxine bound to prealbumin. The second peak was in the albumin region and may represent thyroxine bound to albumin or, more probably, an iodinated protein with approximately the same electrophoretic mobility as albumin in starch gels.

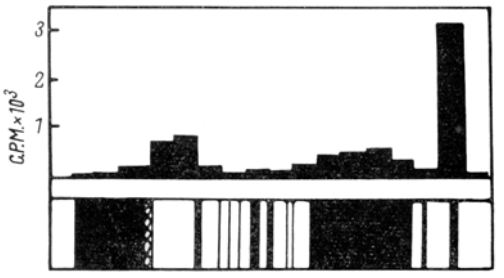

Fig. 7. – Radioactivity in serum proteins of a patient with carcinoma of the thyroid receiving radioiodide therapy, 72 h after administration of 7 mC dose.

Such a protein component has been described by several authors[25] and has been shown to be immunologically distinct from iodinated serum albumin[26]. The third peak of radioactivity near the origin had the same electrophoretic mobility on starch gels as purified human thyroglobulin. Labelling of thyroglobulin in sera of patients receiving radioiodine therapy has previously been demonstrated[27]. On filter paper electrophoresis thyroglobulin migrates in the α-globulin region, and the low mobility in starch gels is presumably due to the relatively large size of the molecules (ca. 660,000)[28].

Acknowledgments. – I am indebted to Dr. R. Oliver, Churchill Hospital, Oxford, for sera of patients receiving tracer and therapeutic doses of radioiodine, and Drs. B. Mallett and S. T. E. Callender, Radcliffe Infirmary, Oxford, for sera from patients receiving radioiron. Dr. P. H. G. Gell kindly supplied purified preparations of caeruloplasmin and an anti-transferrin serum, and Dr. B. A. Askonas purified human thyroglobulin. I am indebted also to Dr. J. Tata for providing labelled thyroxine and triiodothyronine and for helpful discussions, and to Dr. D. R. Bangham for allowing me to quote unpublished observations.

Résumé

On a analysé au moyen d'électrophorèse sur gel d'amidon les protéines qui, dans le sérum humain, lient le fer, le cuivre et l'hormone thyroïdienne, en utilisant comme témoins des protéines purifiées et la radioactivité. Le fer est lié par la β-globuline qui, dans certaines familles, paraît se diviser en deux composants. Le cuivre est lié par la céruloplasmine, qui émigre dans la position α_2 rapide. La protéine principale liant la thyroxine est la préalbumine.

[21] H. E. Schultze, M. Schonberger, and G. Schwick, Biochem. Z. *328*, 267 (1956).

[22] W. G. Gratzer, personal communication.

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