CLXIII. THE ALLEGED PRESENCE OF BILE SALTS IN NORMAL BLOOD.

BY ERNEST WALKER.

From the Department of Biochemistry, Oxford.

(Received August 29th, 1930.)

THE supposition that there might be small amounts of bile salts in normal blood arose from certain considerations which are not pertinent to the present communication. The known association between bile salts and lipoids makes the supposition plausible, particularly when considering how the latter are transported in the blood. Furthermore it is at least conceivable that the liver fails to pick out quantitatively the bile salts in the portal circulation; or that bile salts might find their way into the systemic circulation *via* the lymphatic system. The relatively large amounts of cholesterol and of cholesteryl esters in blood would be expected to counteract any haemolytic effect of bile salts. The present communication deals with the search for bile salts in normal blood.

Several workers have assumed that a positive Pettenkofer reaction given by extracts of normal blood is an indication of the presence of bile salts. Thus Aldrich and Bledsoe [1928] state that bile salts are present in human blood to the extent of 3 to 6 mg. per 100 cc. Charlet [1929] gives 3.5 to 3.8 mg. per 100 cc. in normal rat blood. On the other hand, but still using colorimetric tests based upon the Pettenkofer reaction, Perlzweig and Barron [1926] and Gregory and Pascoe [1929] deny the presence of bile salts in normal blood. It seems, therefore, that the only satisfactory method of establishing the presence of bile salts in normal blood lies in their isolation from extracts of blood.

Some time ago I set out to do this but have been unsuccessful. This work therefore need only be referred to briefly. The blood used was mainly that of the ox, straight from the slaughtered animal. In most cases it was oxalated; but defibrinated blood was also tried. Whole blood was used, the separation into plasma and corpuscles affording no particular advantage, and was treated with five times its volume of 97 % alcohol; other protein precipitants were tried but discarded. The Pettenkofer reaction was applied as follows: 5 cc. of the alcoholic extract were evaporated down on a water-bath to 1 cc.; 2-3 drops of 10 % sucrose solution were added and then 1-2 cc. of strong sulphuric acid were poured carefully down the side of the test-tube. A purple ring formed at the junction of the two layers, sometimes only on standing, and of varying intensity; but all blood extracts tested gave a positive reaction. It was this colour reaction which was followed in the attempts to concentrate the so-called bile salts, and not the more elaborate technique used by Aldrich and Bledsoe

[1928] for the quantitative estimation of bile salts. The volume of alcohol used for the initial precipitation was varied from 3 to 10 without any particular advantage accruing. The subsequent processes of concentration were varied, but in general the aim was to clear the extract, then to concentrate it *in vacuo* and to take up the residue in absolute alcohol. Treatment of such an alcoholic extract with ether resulted in a precipitate which should have contained any bile salts present. In practice it resulted in the (apparent) complete disappearance of the substance responsible for the colour reaction. Neither the precipitate nor the alcohol-ether mixture gave any Pettenkofer reaction. The ether-alcohol mixture was evaporated to dryness, at room temperature, and the residue was extracted with water, dilute alkali and dilute acid respectively; none of these filtered extracts gave a positive reaction. The most puzzling feature of the whole search was that the Pettenkofer reaction appeared to follow the lipoid fraction, whereas it would have been expected to follow the aqueous fraction if it were due to bile salts. This anomaly did not entirely dispose of the possibility of the reaction being due to bile salts as it was always conceivable that the bile salts were present in some sort of association with cholesterol [cf. Reinboldt, 1929; Verzar and Kuthy, 1929], which might well modify their physical properties. When however it was found that treatment of the initial alcoholic extract with neutral or basic lead acetate failed to remove the substance responsible for the Pettenkofer reaction it was felt that the bile salt hypothesis was no longer tenable. It was at this stage that an earlier observation was recalled which seemed to afford an explanation of the positive Pettenkofer reaction. I had noticed that alcoholic extracts of blood which had been allowed to stand for 24 hours or longer almost invariably deposited fine needle-shaped crystals. These were removed and identified as cholesteryl oleate. Now it has been known for many years that oleic acid will give a positive Pettenkofer reaction. Thudichum [1884] mentions it and Mylius [1887] includes it in a list¹ of substances giving a positive Pettenkofer reaction. Any blood extract therefore might be expected to give a Pettenkofer reaction in virtue of its oleic acid content (present as cholesteryl oleate) and there is no doubt in my mind that this substance was responsible for the colour reaction which I obtained during the fractionation of blood extracts. The question arises, can this be the substance responsible for the positive Pettenkofer reaction obtained by Aldrich and Bledsoe [1928] and others? The answer to this question I believe to be in the affirmative. Briefly, the Aldrich-Bledsoe treatment consists in decolorising the alcoholic extract of blood with the minimal amount of charcoal; adding baryta to the filtrate and evaporating carefully to dryness. The dried residue is then extracted three times with boiling dry ether and the test is performed on the residue. The ether extraction

¹ Gregory and Pascoe [1929] state that Mylius gives a list of 150 substances exhibiting a positive Pettenkofer reaction. Some error seems to have crept into their citation. In the paper referred to by them Mylius gives a list of 15 substances, only eight of which give a positive Pettenkofer reaction.

would remove cholesterol and its esters. If however some of the cholesteryl oleate were hydrolysed, during evaporation to dryness in the presence of baryta, barium oleate would be formed and this would not be removed by ether extraction. Barium oleate is insoluble in dry ether; it also gives a positive Pettenkofer reaction.

I have carried out several experiments which indicate that what is suggested above actually happens. These experiments show, firstly, that cholesteryl oleate is hydrolysed, partially at least, by evaporating to dryness its solution in alcohol to which baryta has been added; secondly, that it is possible to isolate barium oleate from blood extracts which have been submitted to the Aldrich-Bledsoe treatment for the colorimetric estimation of their bile salt content.

EXPERIMENTAL.

1. *Hydrolysis of cholesteryl oleate.* Cholesteryl oleate was prepared according to the method of Hürthle [1895]. A warm saturated solution of the oleate in 99 % alcohol was prepared and filtered. 100 cc. of this were taken and used for the estimation of the cholesteryl oleate content. This was found to be 0.1056 g. A second batch of 100 cc. was treated with 2 cc. of saturated aqueous baryta solution and the mixture evaporated carefully to dryness: first on the water-bath and then in a vacuum desiccator. The dried residue was extracted three times with hot 95 % alcohol to remove cholesterol (if formed) and unchanged cholesteryl oleate. The cholesterol content of these united alcoholic extracts, after filtering, was determined by the digitonin method. The mean of two estimations gave a value of 12.36 mg.; this corresponds to hydrolysis of the ester to the extent of 19.72 %. This is not very great; but it should be pointed out that the proportion of baryta to cholesteryl oleate was much lower than obtains in the Aldrich-Bledsoe treatment.

2. *Isolation of barium oleate.* In this experiment blood was submitted, as far as possible, to the process described by Aldrich and Bledsoe [1928] for the quantitative estimation of bile salts in blood. One litre of ox-blood was poured into 7 litres of 97 % alcohol with stirring. After standing overnight it was filtered at the pump and the filtrate (amounting to 5.5 litres) was treated with 13 g. of norite charcoal. After shaking the mixture was filtered and to the filtrate were added 333 cc. of saturated baryta solution. The mixture was then concentrated by distillation *in vacuo*; the final stages of concentration to dryness were effected by warm air, followed by the vacuum desiccator. The residue was later extracted three times with boiling dry ether. The dry residue resulting from this treatment was then extracted with 100 cc. of moist benzene (the only practicable solvent for barium oleate). The extract was filtered and the benzene was driven off at low heat; the residue was ground up under warm absolute alcohol, to remove traces of water (and cholesterol not removed by the ether extraction). The mixture was filtered whilst warm and the filtrate was discarded. The residue, supposedly barium oleate, was dried in a vacuum

desiccator; it was then decomposed with hydrochloric acid under ether and the ethereal layer was separated and evaporated to dryness. The oily residue was converted into its lead salt and further purification was attempted by solution in ether, in which solvent lead oleate is characteristically soluble. Most of the product dissolved in ether on warming; the solution was filtered and the ethereal filtrate was evaporated to dryness. The residue amounted to 7.8 mg. The whole of this was used for a determination of the iodine value; the value obtained being 74.3. A specimen of synthetic lead oleate, similarly treated, gave a value of 76.5. Taking into account, therefore, its solubility in ether, there seems every reason to believe that the product obtained was lead oleate, which means that the substance separated by extraction with moist benzene was barium oleate. The yield was small; but the aim of the experiment was simply to demonstrate that barium oleate was formed during the Aldrich-Bledsoe treatment of blood.

Some reference must be made to the occurrence of cholesteryl oleate in blood. Hürthle [1895] demonstrated its presence in the blood of the dog, horse and rabbit; but he makes no mention of human blood. Nor have I found elsewhere any reference to the identification of cholesteryl oleate in human blood. To settle the question I took 10 cc. of venous blood from a normal subject and precipitated it by treatment with 8 volumes of 97 % alcohol. The filtrate from this mixture deposited fine crystals of cholesteryl oleate within 48 hours (34 mg. per 100 cc. blood). These were identified by their solubilities, crystalline form, colour reaction and melting point.

SUMMARY.

1. No evidence could be obtained of the presence of bile salts in normal blood (ox and sheep).
2. The positive Pettenkofer reaction obtained with alcoholic extracts of blood is attributed to cholesteryl oleate.
3. Suggestions are made which would account for the positive reaction obtained after the residues from alcoholic blood extracts have been extracted with ether.
4. Cholesteryl oleate is readily demonstrable in extracts of human blood.

It is a pleasure to record my indebtedness to Prof. Peters for his helpful criticism.

REFERENCES.

- Aldrich and Bledsoe (1928). *J. Biol. Chem.* **77**, 519.
Charlet (1929). *Biochem. Z.* **210**, 42.
Gregory and Pascoe (1929). *J. Biol. Chem.* **83**, 35.
Hürthle (1895). *Z. physiol. Chem.* **21**, 331.
Mylius (1887). *Z. physiol. Chem.* **11**, 492.
Perlzweig and Barron (1926). *Proc. Soc. Exp. Biol. Med.* **29**, 233.
Reinboldt (1929). *Z. physiol. Chem.* **180**, 180.
Thudichum (1884). Chemistry of the brain.
Verzar and Kuthy (1929). *Biochem. Z.* **210**, 265.