XXXII. NOTE ON THE SEPARATION OF A FAT PIGMENT FROM ACCOMPANYING FAT.

By ARCHIBALD NEIL CURRIE.

From the Research Department, Glasgow Royal Cancer Hospital.

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THE very small concentration of pigment substance in adipose tissue fat has always been the chief factor militating against a concise characterisation of its chemical properties, and the various methods devised for its isolation have been built on a very insecure basis of solubility differentiation from accompanying bodies nearly related in chemical properties.

In considering the problem of fat pigment extraction, one recalls in another direction, the work of Noël Paton and others [1898] on the pigments in the muscles and ovaries of the salmon. Here, the pigment-binding substance, because of its protein nature and its marked difference in chemical properties from the fats, phospholipins and pigment itself, was easily freed from pigment; but adipose tissue pigment—adipochrome, as we may call it—is soluble in practically all the fat solvents, and its separation must ultimately be determined by a decided difference in affinity with a common third substance. This method of separation which I have found very successful in the results it yields will be referred to later.

There are various mechanical means of separating pigment in a tolerable state of purity. In this laboratory, a process of fractional sedimentation in light petroleum was attempted and yielded small amounts. The fat was dissolved in the petroleum and allowed to stand for some time when a sedimentation of brownish-yellow substance took place. The supernatant liquid was removed and fresh portions of pigmented fat were introduced, the solution being once again submitted to sedimentation. Finally the sedimented substance was collected, redissolved in light petroleum and allowed to stand for some time. Sedimentation set in again, and this process of sedimentation and re-solution was carried out until a small sample of pigment almost free from fat was obtained. The disadvantage of this method is that because of the close agreement in the solubilities of adipochrome and fat in light petroleum only a small percentage separation takes place.

Another method attempted was to filter a fat emulsion through a Chamberland candle under a pressure of 150 lbs. per square inch. The earlier filtrates contained a completely colourless fat, but the difficulty experienced in the separation of residual pigment from fat was so great as to render this process

practically valueless. In fact, the final extraction by this method merely resolved itself into an operation of fractional sedimentation the chief advantage of which is that it gives a much higher concentration in pigment as compared with fat than the last method.

Such modes of separation as above outlined refer solely to *in situ* extraction of pigment. More efficient methods depend on hydrolytic treatment of the fat.

The common hydrolytic agents used are caustic soda and caustic potash in alcoholic solution. These agents may be quite efficient for ordinary laboratory work, but the chief defect attendant on their use is that the alkali reacts with alcohol producing in very low concentration a yellowish-red substance (probably a mixture of aldehyde resins) similar in appearance to adipochrome in alcoholic solution. This substance is quite as elusive as adipochrome in its behaviour towards solvents. It is soluble in ether, alcohol, cold and warm benzene, light petroleum, chloroform, carbon tetrachloride, in fact, in all the common fat and adipochrome solvents.

Its presence, then, along with extracted pigment tends to render valueless any fundamental tests, since from the ease with which it is formed it may prove a very active agent in the modification of any of the characteristic reactions of adipochrome.

In the lime process of saponification, the fat can be hydrolysed in a very short time if the autoclave is used. For a fat of molecular weight 890 the amount of lime required to convert the glyceride into glycerol and the calcium salt of the fatty acid is 9.4 % of the weight of glyceride, but in order to complete the hydrolysis under ordinary pressure 12–14 % by weight of lime is required. Lime, however, can be used as a catalyst if the conditions of carrying out the process are modified. At a pressure of eight atmospheres complete hydrolysis can take place with a third of the theoretical quantity of lime required to yield the calcium salt: at twelve atmospheres as small a quantity as 1 % by weight of lime is sufficient to effect complete hydrolysis.

The calcium oxide and the baryta methods were attempted here. It was thought that the Twitchell method (French Patent, 456956) while being of considerable use in the process of commercial hydrolysis [see Henderson, 1919] might in the laboratory give rise to undesirable coloured substances and difficultly separable compounds. This method has not meantime been adopted. The calcium oxide and barium hydroxide methods, however, promise to give good results. Practically, the better results were obtained with calcium oxide. The hydrolysis was quite effective with this compound and the small proportion of calcium salt formed was easily disposed of. In the barium hydroxide method, the glycerol-containing portion of the hydrolysate was found to be quite rich in barium salts of the fatty acids present. These came down easily, but not completely on cooling, bringing pigment substance with them, and its separation was rendered the more difficult because of subsequent acidification.

METHOD.

The method devised for the separation of pigment is illustrated in the following experiment. 200 g. of fat (obtained from the abdominal wall of stall fed cattle) were taken in a 5 litre beaker. About 14 g. of lime were finely powdered in a mortar and intimately mixed with the glyceride. The fatty charge was then covered with distilled water; a piece of well-washed and dried muslin cloth was tied over the mouth of the beaker which was then lowered into the autoclave. A pressure corresponding to a temperature of 140° was maintained for ten hours, and at the end of that time complete hydrolysis had taken place. The liquid subjacent to the fatty acids was removed (through muslin cloth tied to mouth of beaker), filtered, and was found to be straw-yellow in colour. Further extractions were made from the fatty acid with hot distilled water until the extracts were no longer coloured. These were added together in a large beaker and after water had been added were concentrated to a small bulk. A large quantity of water was used in order to dissociate as far as possible the calcium salts of fatty acids present in small quantity in the glycerol-containing aqueous extract. The concentrate was distinctly alkaline in reaction to litmus and in its alkaline state was extracted with ether. The ether layer contained very small quantities of fatty acid dissolved out in the long continued extraction. The yellow pigment substance was entirely taken up by the aqueous layer as it was quite insoluble in ether. The aqueous layer was then drawn off into a second separating funnel and the top ethereal fatty acid-containing layer was discarded. In this way pigment was separated from fatty acid. The aqueous layer still contained glycerol. This yellow layer was acidified with 20 % hydrochloric acid. There was very little change in colour on acidification, but after ether had been added and the contents of the separating funnel shaken up, the yellow colouring matter was completely transferred to the top layer.

In this way the pigment was freed from glycerol. These two simple operations effect a separation of pigment from both fatty acid and glycerol, and the difficulties associated with the other methods of hydrolysis, *e.g.* (1) the development (with alcoholic caustic potash) of an independent coloured substance which manifests the same physical peculiarities as regards solubility as the adipochrome, and (2) the almost inseparable association of adipochrome with fatty acid on account of their near affinity for the common fat solvents, were completely eliminated. It was surmised that adipochrome behaved as an acid substance and was stronger, *i.e.* dissociable to a higher degree than stearic or palmitic acid. It seems to form a stable union with barium hydroxide, calcium hydroxide, generally with the alkaline hydroxides and several other metallic oxides. Calcium hydroxide effects a rather stable union with the adipochrome "acid" substance. This stable union resists hydrolytic dissociation to a marked degree. For example, the barium and calcium salts of stearic acid (insoluble in water) undergo dissociation to such

an extent that they give up their bases on washing with water. The above fact bears on this method of extraction, as the pigment "salt" is retained in the first water layer along with the calcium hydroxide from the broken down calcium stearate while the stearic acid present in very small quantity goes to the ether layer. In this way adipochrome "salt" is separated from fatty acids and their calcium salts. It is soluble in water and in alcohol, but is insoluble in ether, as shown above. On acidification with 20 % hydrochloric acid, or even 10 % tartaric acid solution there is an immediate migration of pigment substance to the ether layer showing that adipochrome which has been freed from combination with calcium is soluble in ether.

The scheme of separation is shown below. The chemical nature of adipochrome will be discussed in a subsequent paper.

REFERENCES.

- Henderson (1919). *Catalysis in Industrial Chemistry*.
 Paton, D. Noël (1898). Report of Investigations on the Life History of the Salmon. *Fishery Board of Scotland*, No. 28230.