A Contribution to the Chromatology of the Bile. By
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One of the chief biliary pigments is bilirubin. It has a red-orange colour, and is derived from the decomposition of hæmoglobin. It can be oxidised first into a green, then into a blue, then into a red, and finally into a yellow-brown pigment. Between the blue and red a violet substance is produced, but it is uncertain whether or not this is only a mixture of the blue and red pigment. The formation of the green pigment, according to Städeler,* is due to oxidation, together with the addition of a molecule of water. These pigments do not always present exactly the same characters. Thus, according to Dr MacMunn, the green pigment present in the ox-bile differs from what is artificially produced from the oxidation of bilirubin, say, from human bile, in that it is soluble in chloroform.† This pigment can, however, be oxidised up into the blue pigment, and so on; and it belongs, therefore, to what we may term the bilirubin series. Its colour indicates its position in the scale of oxidised products. We shall in this paper use the term biliverdin as designating a green pigment, which is more oxidised than the bilirubin, and bilicyanin as designating a blue pigment still more oxidised. The violet substance (if such exist) we shall term the violet pigment, the red oxidation product the red pigment, and, finally, the yellow-brown pigment, the most highly oxidised product of all, choletelin.

Although, by the action of an oxidising agent, such as impure nitric acid, it is easy to pass from a lower to a higher member of the bilirubin series, it is frequently, and we believe truly stated, that no successful attempt has been made to reduce the higher back again to the lower ones. By the reducing action of, say, sodium amalgam, hydrobilirubin ($C_{32}H_{40}N_4O_7$) has been produced both from bilirubin and from biliverdin. When, however, ox-bile is placed in a tall vessel, and allowed to remain for some hours, we have observed that a change in colour takes place. If blue pigment is present

* Gorup-Besanez, *Physiol. Chemie*, p. 207.

† *Jour. of Phys.*, vol. vi. p. 2.

it changes to green, and this finally to orange-brown. If some of the brown bile be then treated with a few drops of nitric acid, it is oxidised back into biliverdin, and further additions of acid develop the blue, violet, red, and yellow pigments. Here, then, without going any further, we have an instance of the reduction of the pigment. The reduction does not produce hydrobilirubin alone, as that substance cannot be reoxidised in the way we have described.

If a bladder, fresh from the slaughter-house, be opened, one seldom or never fails to see signs of reduction within it. Blue or blue-green bile fills the cavity, but thick orange-brown bile is seen next the mucous membrane, which is itself brown in colour. It is not improbable, therefore, that during life, reduction of the biliverdin takes place, due perhaps to the action of the mucous membrane of the gall-bladder or to the mucus secreted by it. This is, indeed, almost certainly the case, and one can recall the fact that the pigment present in gall-stones from the ox consists, not of biliverdin, but of bilirubin.

It was obviously a matter of some importance to investigate the reduction processes we have just described, in order, if possible, to ascertain their cause, and the influence of modifying conditions upon them. We have been greatly assisted in this inquiry by Dr MacMunn. We sent to him on several occasions solutions in which we had difficulty in determining whether definite absorption-bands were present, and we have very warmly to thank him for the courtesy with which he was ever willing to help us.

Experiment I.—Temperature of the laboratory, 60°. Two test-tubes were filled with fresh green ox-bile, and watched from day to day. After three days the bile in the lower part of one of the tubes had become of a brown-green colour. The change in colour spread upwards, and in twelve hours the whole tube contained bile of a uniform brown tint. Putrefaction, indicated by an unpleasant odour, had set in by this time. In the other tube the bile did not change in colour, nor was there any sign of putrefaction until the following day, when both appeared simultaneously. Later on the bile became by degrees of a light amber colour, giving, however, the play of colours for nearly four months, after which Gmelin's test failed. The fluid was then examined with the spectroscope. There was slight shading at the violet end of the spectrum, and the

bands of cholo-hæmatin* were present. There was no distinct hydrobilirubin band. It is seen, therefore, that on exposure to the atmosphere the biliverdin is reduced; the bilirubin disappears without forming hydrobilirubin.

Experiment II.—Test-tubes were filled with ox-bile, and pieces of the mucous membrane of the gall-bladder were added. The changes observed were similar to those detailed in Experiment I. The mucous membrane seemed to hasten the reduction of the bile in its neighbourhood. The reduction of the pigment seemed remarkably to coincide in point of time with the establishment of putrefaction. The following experiments were conducted in order to eliminate putrefactive changes from the other conditions present.

Experiment III.—Ox-bile was boiled in test-tubes which had previously been plugged with wool. No alteration in colour was produced by boiling, nor did any take place for seven days. It then became of a light brown colour, rapidly fading, until after a fortnight it was nearly colourless. Gmelin's test failed after three weeks, and two weeks afterwards the fluid was examined with the spectroscope. Cholo-hæmatin bands were absent; there was possibly a trace of hydrobilirubin. This experiment indicates that reduction of the biliverdin takes place, and the bilirubin disappears in bile which has been prevented from putrefying. The reduction of the biliverdin seems to be hastened by putrefaction.

Experiment IV.—Ox-bile was evaporated to dryness at 50°. It very slowly changed its colour, and only failed to give Gmelin's test after several months' exposure.

Experiment V.—Some exhausted and sterilised glass tubes were drawn out into capillary points. The points were thrust through the wall of a fresh gall-bladder, and broken off by the fingers which grasped them from without the bladder. They instantly filled with bile, and after their withdrawal from the bladder their ends were at once sealed up in the blow-flame. No change of colour occurred for fourteen days; the weather was cold and dull. By that time, however, they had become brown in colour. No further change occurred, and after a year they gave a distinct play of colours with nitric acid.

* Cholo-hæmatin is a pigment which gives absorption bands. It is present and often partially replaces biliverdin, in ox-bile.—MacMunn, *loc. cit.*

Changes, not very dissimilar in their nature, occur when blood is received directly into a sterilised tube from an artery. In this case the oxyhæmoglobin is reduced, and remains for years without undergoing further change. In the sterilised tubes filled with bile the change in colour seemed to be influenced in the thick mucus of the bladder, for those that contained most mucus invariably became brown before those that contained less mucus.

It will be seen, however, by the results of the next two experiments, that reduction occurs quite readily in the absence of bladder mucus. The two experiments were unfortunately performed, the first in dull cold weather, and the second in warm bright weather, so that they cannot be very rigidly compared. One can conclude, however, from the general results obtained, that thick bladder mucus assists the reduction, although its presence is not essential.

Experiment VI.—Exhausted and sterilised tubes were filled with bile drawn directly from the hepatic duct of a freshly killed ox. Reduction took place in three days.

Experiment VII.—In order to ascertain whether reduction would take place in bile absolutely free from mucus, this substance was precipitated by means of alcohol. The filtrate subsequent to the separation of the mucus was evaporated over a water-bath until the alcohol was expelled. The reduction took place, however, in two days; the weather was hot and bright.

The last experiments were made during hot midsummer weather, and it was noticed that, while in all cases reduction took place with rapidity, this was especially the case in the tubes most exposed to light. In the following experiments the action of light was more fully investigated.

Experiment VIII.—(a) Bile within test-tubes exposed to light was reduced in twenty-four hours, and after a week failed to give Gmelin's test; weather hot and bright. Other portions of the same bile in a dark metal chamber were reduced in three days.

(b) Bile boiled in plugged tubes and exposed to light was reduced in twenty-eight hours. A portion treated in a similar way, but placed within the dark chamber, still preserved a trace of the original green colour for seventeen days.

(c) Bile within the exhausted and sterilised tubes was reduced

by the light in twenty hours. In the dark chamber they began slowly to undergo change at the bottom of the tube after twenty-four hours.

- (d) A film of bile about 1 millimetre thick was dried at a low temperature. On the fourth day it had completely changed its colour to brown; while, on the other hand, a film, similarly prepared, but kept in the dark, remained blue-green, the original colour of the bile, for a year and a-half—the time when it was finally examined.

It would seem, therefore, that biliverdin readily parts with its oxygen like oxyhæmoglobin. It is reduced in the sterilised tubes to bilirubin, but no further. This reduction is hastened by light, and putrefaction, and the presence of thick mucus. It is only prevented by drying the bile and keeping it in a dark chamber.

When bile putrefies, or when, without putrefaction, the bile has been altered by boiling it, the bilirubin finally disappears, and no play of colour is obtained by the application of Gmelin's test.

This residue contained a brown pigment giving no absorption bands, and differing from hydrobilirubin in its solubilities. It was insoluble in ether, but readily soluble in alcohol.

APPENDIX.

On the Reduction and Oxygenation of Pigments in the Bilirubin Series.

Bilirubin, when exposed to the air, under ordinary circumstances, never becomes converted into biliverdin. This takes place, however, if the solution has previously been rendered strongly alkaline by the addition of caustic soda solution. Both nascent oxygen and ozone, we find, are capable of effecting this change in bile of normal reaction. To do this experiment, pieces of blotting-paper soaked in bile should be exposed to the vapour of ozone; ozonic ether, liberating nascent oxygen, can also oxidise these papers. If human bile is poured into a small beaker, and a stream of electricity passed through it, obtained from some five or six Grove cells—the terminals should be of platinum—the oxygen given off from the positive terminal, in the course of three or four minutes, changes the bile in its vicinity, first to a green, and finally to a blue-green

colour. The experiment may be performed in, perhaps, a more satisfactory manner by dipping a piece of blotting-paper in the bile, and laying it directly on the terminals. The brown colour changes near the positive terminal, first to green, then to blue, and finally to violet. At this stage, however, much of the pigment becomes bleached, so that the violet is not so distinct as either the blue or the green.

The lower oxidation products of bilirubin can be reduced artificially. If some bile be acidulated with impure nitric acid, so as to oxidise its bilirubin to biliverdin or bilicyanin, and if pieces of blotting-paper be then dipped in these, the vapour of ammonium sulphide can reduce them. The blue colour is reduced through green to brown, and it can then be oxidised by nitric acid. If blotting-paper is soaked in bile and reoxidised by the positive pole of the battery, the pigment is again reduced on reversing the poles. The brown can be oxidised to green and then to blue, and afterwards reduced to brown, passing through green. If oxidised to red, reducing reagents then change the colour to a yellow-brown, but not through any intermediate stages. Oxidising agents restore the original red colour.

It is much easier to perform these experiments with bile than with pure bilirubin. Bilirubin dissolved in caustic soda is difficult either to oxidise or to reduce. Pure bilirubin, powdered on blotting-paper, moistened with normal saline solution, can be oxidised and reduced without difficulty by the current given by three or four Groves.

On the Identity of Hofmann's "Dibenzyl-Phosphine" with Oxide of Tribenzyl-Phosphine, and on some other Points connected with the Phosphorised Derivations of Benzyl. By Professor Letts and R. F. Blake, Queen's College, Belfast.

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In his well-known researches on the phosphines, Hofmann has shown (or believes that he has shown) that when an alkyl iodide (or other haloid derivative) is heated with phosphonium iodide and oxide of zinc, primary and secondary phosphines alone result; whereas, when an alcohol is heated with iodide of phosphonium, tertiary and quaternary phosphines are formed exclusively. Thus