XLI. NOTE ON A SOURCE OF ERROR IN THE COLORIMETRIC METHODS FOR THE ESTI- MATION OF CHOLESTEROL IN TISSUE FATS.

BY JOHN ADDYMAN GARDNER
AND FRANCIS WILLIAM FOX (Beit Memorial Fellow).

From the Physiological Laboratory, University of London.

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IN the course of an investigation of the effect of heating the amorphous sterols of human faeces in sealed tubes with alcoholic potash, it was noticed that alcoholic potash which had been heated alone in a blank experiment, when diluted with water and extracted with ether, yielded to the ether a small quantity of resinous matter, which on treatment in chloroform solution with acetic anhydride and a few drops of sulphuric acid gave a marked brown colour which on standing developed a yellowish-green tint. A standard alcoholic potash solution, that had stood in the laboratory for several years and was brown in colour, after extraction and treatment in a similar manner gave a brownish-red colour which changed in a few minutes to a marked green. Another solution which was many years old and almost black in colour gave a brownish-red colour which on standing developed a slight green tinge. The same solution, however, boiled under a reflex for two hours before extraction, gave a deep brown colour, but no green tint developed. On heating 5 grams of potash with 25 cc. of methylated spirit in a closed tube at 110° for several hours a dark brown solution was obtained. This after dilution and extraction with ether yielded a brown resin, which in chloroform solution gave with the above reagents a deep reddish-brown colour, and on standing developed a strong green fluorescence. It was red by transmitted light. A specimen of aldehyde resin made by the action of aqueous soda on acetaldehyde gave a cherry-red colour, but no green. Since in estimations of the sterols of tissues by colorimetric methods the fats are hydrolysed by boiling with alcoholic potash or soda or sodium ethoxide, and in some methods of extracting fatty matter the whole tissue is destroyed by alcoholic soda, it seemed important to submit the above observation to further investigation.

The alcohol used was the duty-free non-methylated alcohol of commerce of good quality as usually supplied to laboratories. In one series of experiments measured volumes of alcohol were heated with weighed quantities of powdered caustic soda or potash in sealed tubes at 110° for 2 to 4 hours. In

another series the alkali and alcohol were heated for several hours on a water-bath in a long-necked matrass, and in a third series weighed quantities of sodium were dissolved in measured volumes of alcohol and the solutions allowed to stand some time at the laboratory temperature, or in some cases were warmed on the water-bath. The experiments were done in glass vessels and no corks or other substances which might yield soluble matter to the alcohol were used. In most cases very pale yellow solutions were obtained. These were diluted with water and extracted with ether. The ethereal solutions were then thoroughly washed by shaking with water and the ether evaporated. The pale yellow residues, which rarely exceeded a few milligrams per cent. of alcohol, were then dissolved in 5 cc. chloroform and treated with 2 cc. of acetic anhydride and 6 drops (0.4–0.5 cc.) of sulphuric acid. In every case a marked reddish-brown colour was produced, the depth depending on the amount of alcohol used. When a less quantity of acetic anhydride was used a brown precipitate sometimes separated, this however readily dissolved on adding a little more acetic anhydride. The brown solutions on standing sometimes remained unchanged, but in most cases after a few minutes' standing developed a distinct greenish tinge. In a few cases the brown colour became definitely a dull grass green. The green tinge faded on standing overnight, but the brown colour was more permanent. The minute residues left on evaporating quantities of alcohol of the order of those used in these experiments gave no appreciable reaction.

It seemed likely that these effects were due to traces of aldehydes and other similar impurities in the alcohol, and in order to ascertain whether these could be conveniently got rid of by boiling with alkali, 1 litre of alcohol was boiled in a matrass with 200 grams of caustic soda for 4 hours. The alcohol was then distilled off.

50 cc. of this distillate were heated in a sealed tube with 2 grams of caustic soda for 4 hours at 110°. The solution became pale yellow. After extraction with ether a trace of residue was obtained. This was dissolved in 5 cc. chloroform and treated with 2 cc. acetic anhydride and 6 drops of sulphuric acid. The liquid became pale brown and on standing for a few minutes changed to a greenish brown. The colour was quite different from the blue-green colour given by cholesterol, but as far as we could judge the body of colour was about the same as that given by a standard cholesterol solution containing 0.0009 gram sterol.

In another experiment 50 cc. of the distillate were boiled with 2 grams of caustic soda on the water-bath for 4–5 hours. The solution was bright yellow. On extraction 1.4 mg. of residue was obtained which was tested as above. A dusky brown solution was obtained, but it did not turn green. A specimen of alcohol which had been dried by boiling for many hours with a large quantity of quicklime, and then distilled, but which had stood in the bottle for some weeks, was also tested. 50 cc. were heated in a sealed tube with 2 grams caustic soda at 110° for 4 hours. A yellowish solution was left. This on

extraction yielded a slight residue, which on testing gave a well-marked brown colour which gradually changed to a definite dull grass green. On comparing as well as we could with a standard cholesterol we concluded that the colour was about equivalent to 1.3 mg. of cholesterol.

A control experiment by evaporating 50 cc. to dryness gave no reaction and similarly in the case of the ether used. Whether these colour reactions are entirely due to impurities, or whether they are given by chemically pure alcohol has not been investigated.

It is obvious, however, that the production of any green colour due to alcohol used even though of a different shade would introduce an error into the colorimetric estimation of cholesterol. But even when no green tint is produced the brown colour so modifies the quality of the colour due to the cholesterol as to make it very difficult to compare with that of the standard cholesterol solution with any degree of precision. This effect can be readily demonstrated by treating a chloroform solution of pure cholesterol of suitable strength with acetic anhydride and sulphuric acid and when the green stage has been reached dividing into two equal parts. If one is then diluted with an equal volume of one of the above mentioned brown solutions with an approximately equivalent body of colour, and the other with a mixture of chloroform and acetic anhydride of similar concentration the difference can be readily seen. The one is a yellowish or grass green and the other a blue green.