THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL.

II. A SEPARATION INTO AN ACTIVE AND AN INACTIVE FRACTION.

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(Received for publication, June 29, 1926.)

As is well known, young rats can be regularly protected from rickets by the addition to their dietary of 1 mg. or less of irradiated cholesterol. Various experiences led us to believe that this cholesterol was a mixture of active and inactive material and that an attempt should, therefore, be made to fractionate it. In the first place prolonged irradiation gradually inactivates cholesterol which has been rendered antirachitic by means of ultra-violet rays. According to our conception of the process, cholesterol in the course of irradiation is being inactivated at the same time that it is being activated, and thus represents a mixture of at least two forms of cholesterol. Another experience which induced us to undertake fractionization experiments was the recent report of Beumer (1) to the effect that activated cholesterol could be separated into a digitonin-precipitable and non-precipitable substance. His tests did not include an investigation as to whether the non-precipitable residue of irradiated cholesterol possessed antirachitic potency. As reported in a recent communication (2), an attempt was made to obtain an active fraction of irradiated cholesterol by means of the well known method of precipitation with an alcoholic solution of digitonin. The filtrate was evaporated to dryness in vacuo, the white residue taken up and washed with small volumes of ether, and the solution then evaporated in vacuo. This amorphous residue, as well as the digitonin precipitate, was fed to series of rats, but much to our surprise neither
fraction showed any protective power. Several experiments of this kind were carried out without success.

Recently we have modified the technique of fractionization in several particulars; the separation has been carried out in an atmosphere of nitrogen, the oily menstruum in which the fractions are suspended was mixed immediately with the fractions, and curative rather than prophylactic tests were employed in order to shorten the period of the tests. This curtailment was resorted to with the idea that our failure to obtain protection might have resulted from a rapid deterioration of the fraction. The method was as follows:

The separation of the cholesterol digitonide from the soluble fraction was carried out in an atmosphere of nitrogen, using a modification of Steinberg's apparatus for suction filtration. After thorough washing of the precipitate, linseed oil was run directly into the receiving flask and the alcohol distilled off in vacuo. Previous experiments show that the active fraction so obtained is about 4.5 per cent of the total irradiated cholesterol.

The precipitated digitonide was mixed with linseed oil on the filter plate before transferring to a flask. Because of the very limited solubility of this fraction in the oil, it is obvious that exposure to oxygen was not avoided.

When series of rats were fed the digitonin-precipitable and the non-precipitable fractions prepared in this way, a clear cut result was obtained; those animals receiving the former fraction showed no subsequent healing of the rachitic process, whereas those which received the non-precipitable fraction gave evidence of marked healing within the experimental period of 9 days. Table I shows that the percentage of inorganic phosphate of the blood was, as would be expected, definitely higher in the latter group. The active fraction constituted only about 4 to 5 per cent of the original amount of cholesterol which had been irradiated for a period of 1 hour at a distance of 1 foot.

This experiment is of interest as providing a method of concentrating activated cholesterol, as well as indicating that only a small part (approximately 5 per cent) of activated cholesterol possesses antirachitic properties. Its greater interest, at the present time, would seem to lie in the fact that the result links the specific antirachitic power of activated cholesterol with that
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of cod liver oil. As is well known, the antirachitic potency of cod liver oil has been found to be due entirely to its non-saponifiable fraction. Furthermore, it has been shown by Dubin and Funk (3) that this fraction can be rendered still more potent by means of "eliminating the cholesterol" by precipitation with digitonin. Coupling these fractionization experiments of cod liver oil and of activated cholesterol, we have good evidence to the effect that a close chemical similarity exists between the active principle of these two substances, and that their protective and curative action in rickets is due to a factor common to both. Probably the activity of cod liver oil is to be ultimately ascribed to ultra-violet radiation either directly of the cod itself, or more probably, indirectly through the food.

In a short communication Koch, Cahan, and Gustavson (4) have reported recently an experiment in which, "The non-saponifiable fraction of cod liver oil was extracted with liquid ammonia and again a brown, gummy residue was obtained. This fed in daily doses of 2 mg. prevented rickets entirely." In view of this result we carried out a series of experiments to ascertain whether activated cholesterol could be fractionated similarly by means of liquid ammonia. The process was as follows:

Anhydrous liquid ammonia was run into a reaction cylinder containing irradiated cholesterol and allowed to remain in contact with the substance, under pressure from the ammonia tank, for 3 hours. After that period the ammonia solution was forced through an asbestos filter and run slowly into a large volume of distilled water. The entire system was flushed thoroughly with ammonia from the tank. The water containing the ammonia-soluble fraction was evaporated nearly to dryness. Linseed oil was then added and the water driven off completely. About 96 per cent of the original amount of irradiated cholesterol was recovered from the reaction chamber. This confirms an ammonia-soluble fraction of somewhat less than 4 per cent, as indicated by direct weights in preliminary experiments. Quantitative determinations were not made.

Rats which were given this preparation in daily amounts of 2.5 mg. were protected from rickets while fed on the standard low phosphorus diet. This result furnishes once more an analogy
between the active antirachitic substance in cod liver oil and that in cholesterol which has been treated with ultra-violet radiations.

Liquid ammonia has been employed successfully by these investigators in obtaining an active fraction, the female sex or ovarian hormone, from follicular fluid and other tissue. This hormone bears certain resemblances to activated cholesterol; both are lipoids of high molecular weight, both unsaturated compounds, and show a high degree of thermostability. In view of these points of similarity, it was thought worth while to investigate whether the female sex hormone possessed any antirachitic properties and also whether irradiated cholesterol showed any of the specific activity of the sex hormone. The fraction obtained from activated cholesterol by means of liquid ammonia was tested by Dr. Robert Frank, but failed to bring about contractions of the uterus when injected subcutaneously in doses of 2.5 mg. This amount of the extract of ovarian follicular fluid suffices to bring about this characteristic reaction. Furthermore, the fraction containing the ovarian hormone, which had been extracted from follicular fluid, shows no antirachitic properties when tested on rats. It is evident, therefore, that in spite of various chemical points of similarity, these two factors are quite different in their essential properties.

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