The Possible Role of Coenzyme A in the Biosynthesis of Cholesterol in the Rat

By G. S. BOYD
Department of Biochemistry, University of Edinburgh

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With the aid of isotopes it has been established by *in vivo* experiments that mammals can synthesize cholesterol from acetate (Bloch & Rittenberg, 1942); and by the tissue-slice technique various mammalian tissues have been shown to be capable of cholesterol biosynthesis from acetate *in vitro* (Bloch, Borek & Rittenberg, 1946; Srere, Chaikoff, Treitman & Burstein, 1950). In such *in vitro* experiments it has been demonstrated that, in this biosynthesis, both carbon atoms of acetate are extensively incorporated into both the *cyclo*pentenophenanthrene ring system of the molecule, and into the *iso*octyl side chain (Bloch & Rittenberg, 1942; Bloch, 1951).

Nevertheless, there still exists a huge gap in our knowledge regarding the precise steps involved in the use of acetate in the biosynthesis of this complex C_{27} molecule. To date, possibly the only established intermediate in this process is acetoacetate, as it has been shown by *in vitro* studies that this compound could be incorporated into cholesterol without prior degradation to acetate (Brady & Gurin, 1951). This work suggests, therefore, that a stage in the biosynthesis of cholesterol might involve the condensation of two acetate molecules.

It has been known for some time that co-factors are required in simple biological acetylation reactions such as the acetylation of choline (Feldberg & Mann, 1945; Lipton & Barron, 1946; Nachmansohn & Berman, 1946) or in the acetylation of a foreign amine, for example sulphanilamide (Lipmann, 1945), while the co-factors necessary in the acetylation of both alcohols and amines were shown to be identical by Lipmann & Kaplan (1946). Soodak & Lipmann (1948) showed that this coenzyme was also involved in the reaction acetate plus acetate yielding acetoacetate. Lipmann, Kaplan, Novelli, Tuttle & Guirard (1947) demonstrated that this co-acetylase factor (coenzyme A) contains pantothenic acid in a bound form. This coenzyme was isolated in fairly pure form by Lipmann, Kaplan, Novelli, Tuttle & Guirard (1950) and its structure established by the joint efforts of many workers including Lipmann *et al.* (1950); Novelli, Kaplan & Lipmann (1950); Baddiley & Thain (1951*a*, *b*, *c*).

According to Lynen, Reichert & Rueff (1951), coenzyme A possibly participates in biological acetylations by accepting an acetyl radical at its terminal sulphydryl group, producing a thioacetyl coenzyme and transferring this 'energy-rich' acetyl group to the substrate.

It has been shown that the coenzyme-A content is lower than normal in tissues obtained from a bird or mammal which has been fed a diet deficient in pantothenate (Olson & Kaplan, 1948; Olson & Stare, 1951) and consequently the ability of an animal to effect acetylations of the types previously mentioned is markedly reduced in this deficiency state.

It seemed of interest to attempt to establish whether the multiple utilization of acetate by mammals in the biosynthesis of cholesterol requires the participation of coenzyme A and this paper deals with the experimental details and implications of work on this problem previously reported in the form of an Abstract (Boyd, 1952).

METHODS

Preparation of animals. The animals used in this study were male and female albino rats of the Wistar strain. After weaning, the rats were placed in wire-mesh metabolism cages in groups of four to six, and allowed tap water *ad libitum*. The diet consisted of approx. 10 g./day/rat of the following basal ration: 23% vitamin-free casein, supplemented with 0·2% cystine; 72% carbohydrate (glucose or sucrose); 5% salt mixture (Phillips & Hart, 1935) containing 0·05% $CoCl_2$, $6H_2O$.

Water-soluble vitamins were added to the diet in the following amounts (per 100 g. basal ration), which are recognized as adequate for the growing rat: thiamine hydrochloride, 500 μg.; riboflavin, 800 μg.; nicotinamide, 4 mg.; pyridoxine hydrochloride, 500 μg.; biotin, 40 μg.; folic acid, 200 μg.; menaphthone (2-methyl-1:4-naphthaquinone), 500 μg.; inositol, 10 mg.; *p*-aminobenzoic acid, 10 mg.; choline chloride, 20 mg.; calcium pantothenate, 3 mg.

The pantothenate-deficient groups, who were litter mates of the control groups, received the diet exactly as above except that they were deprived of pantothenate. All groups received 1 ml. cod-liver oil/5 rats/week, in two divided doses, which is sufficient to supply the fat-soluble vitamins and the 'essential' fatty acids.

Where certain groups of rats received neutral fat (olive oil) in the diet, this was added at a 10·5% level, while a corresponding reduction was made in the carbohydrate content in order to keep the diets isocaloric. Thus in the experiments to be reported in this paper, there were four groups of rats on the following diets: (*a*) fat-replete, pantothenate-replete, (F+, P+); (*b*) fat-replete, pantothenate-deficient, (F+,

P −); (*c*) fat-deficient, pantothenate-replete, (F −, P +); (*d*) fat-deficient, pantothenate-deficient, (F −, P −).

The caloric intakes of all groups were matched day by day; the animals were fed twice daily, at 9 a.m. and 9 p.m., and weighed each morning.

The pantothenate-deficient group showed all the external symptoms of this deficiency state, such as thinning of fur and porphyrin-stained whiskers, etc.; furthermore, after 10 days on this regimen the animals had virtually ceased growing. Selected pantothenate-deficient animals were then given calcium pantothenate in their diet, whereupon they resumed normal growth and were soon free from all deficiency symptoms; these 'test animals' were then discarded.

The growth curves of two groups of animals (F −, P + and F −, P −) are shown in Fig. 1, in which the response of the pantothenate-deficient animals to this factor is shown.

After 40 days on one of the diets previously specified, each animal was anaesthetized with Nembutal (approx. 0·05 ml./100 g. body weight) and the carotid artery exposed. As much blood as possible was removed by cannulation of this vessel into a centrifuge tube containing a minimum of powdered potassium oxalate.

Plasma cholesterol estimation. Samples of the plasma (1 ml.) were extracted with about 15 ml. boiling acetone : ethanol (1:1) in a 25 ml. volumetric flask for a few minutes. The extracts were then cooled, made up to the mark and filtered. Portions of this solution were taken for estimation of total and free cholesterol by the Sperry-Schoenheimer digitonin procedure as modified by Sperry & Webb (1950).

Liver cholesterol estimation. After the death of the animal by exsanguination, the liver was completely removed, blotted on filter paper and weighed. This tissue was ground with an equal wt. of distilled water in an all-glass homogenizer (Potter & Elvehjem, 1936), samples of the suspension were extracted with $CHCl_3$: methanol (1 : 1, v/v) and cholesterol was estimated in exactly the same manner as in the plasma cholesterol determination.

Liver coenzyme A. Samples of the liver suspension were delivered at once into about 30 ml. boiling distilled water in a Pyrex centrifuge tube, and the resulting suspension was kept at boiling point for one minute. The tube was then chilled in ice-water, centrifuged, the supernatant decanted into a 50 ml. graduated vessel and made up to volume with distilled water. These aqueous extracts were stored at −10° until the coenzyme-A assays could be performed. The assay procedure employed was that of Handschumacher, Mueller & Strong (1951), which was checked against the original sulphanilamide method of assay (Lipmann, 1945; Kaplan & Lipmann, 1948) and found to be more rapid than the latter, but yielded similar results. Standard curves, prepared by the use of reference coenzyme-A samples, were used to measure the coenzyme-A concentrations of the aqueous liver extracts (Kaplan & Lipmann, 1948).

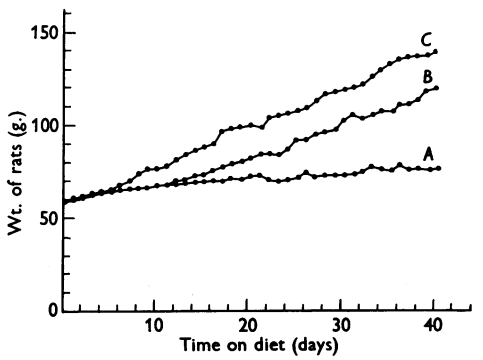


Fig. 1. Growth curves of groups of rats on diets as follows: *A*, pantothenate-deficient; *B*, pantothenate-deficient for 10 days and then pantothenate-replete; *C*, pantothenate-replete.

RESULTS

The results of these observations are summarized in Table 1. When pantothenate was withheld from the diet of a group of young rats for 40 days, the coenzyme-A content of the livers of these animals dropped to a value less than half that found in the control groups of pantothenate-replete animals.

In all four groups of animals dietary restriction failed to alter significantly the level of free cholesterol in the plasma, but the free cholesterol of liver in one group (fat-deficient, pantothenate-deficient) underwent a slight decrease.

If the two fat-deficient groups are compared, it is seen that withholding pantothenate from one group

Table 1. *Comparison of plasma and liver cholesterol, and liver coenzyme* A *in control and pantothenate-deficient rats* (*means* ± S.D.)

Diet	Days on diet	No. of rats	Plasma cholesterol (mg./100 ml.) Total	Free	Ester	Liver cholesterol (mg./100 g. wet wt.) Total	Free	Ester	Liver cholesterol (mg./liver)	Liver coenzyme A (units/g. wet wt.)
Fat-deficient, pantothenate-replete	40	12	64±8·0	17±2·3	47±6·0	248±8·0	183±14	65±14	22·2±2·3	109±17
Fat-deficient, pantothenate-deficient	40	12	44±5·9	16±2·2	28±4·6	181±13	168±10	13±8	16·1±1·8	49±19
Fat-replete, pantothenate-replete	40	12	68±7·7	18±3·5	50±4·7	368±73	205±13	163±63	42·5±7·1	123±21
Fat-replete, pantothenate-deficient	40	12	71±10	18±4·0	53±6·4	301±36	203±8·0	98±31	28·6±3·9	41±8

resulted in a significant drop in the plasma and liver total cholesterol. This drop was due almost entirely to a decrease in the ester cholesterol fraction, since the free cholesterol is fairly constant. Thus on a fat-deficient diet, when the coenzyme-A content of the liver (and presumably other organs) is decreased, the plasma and liver ester cholesterol concentration is reduced.

On the other hand, in the fat-replete groups, the plasma and liver total-cholesterol levels were unaffected by the presence or absence of pantothenate in the diet. Thus the animals in the fat-replete, pantothenate-deficient group maintained normal plasma and liver ester cholesterol levels despite a reduction in tissue coenzyme-A concentration.

DISCUSSION

On a fat-free diet the rat appears to be able to maintain its plasma ester cholesterol at about the normal value, although the liver ester cholesterol concentration is decreased. If fat and pantothenate are withheld from the diet, then the plasma ester cholesterol is markedly reduced and the liver ester cholesterol virtually disappears; in most rats in this group, the livers were completely devoid of any ester cholesterol. Since the animals were maintained on a sterol-free diet, nearly all the carcass cholesterol must have been of endogenous origin. Accepting the normal glycolysis mechanism, presumably most of the acetate used in the biosynthesis of cholesterol arose from glucose. Thus on the fat-free diet, pantothenate restriction resulted in a lowered tissue coenzyme-A concentration, producing a decrease in the plasma and liver ester-cholesterol concentration.

On this basis it would be tempting to suggest that the biosynthesis of cholesterol ester involved coenzyme A.

When neutral fat was incorporated in the diet, however, restriction of dietary pantothenate had no effect on the plasma ester cholesterol concentration, and little, if any, effect on the liver ester cholesterol concentration. It appeared, therefore, that the presence of neutral fat in the diet could overcome the apparent inhibition of ester cholesterol synthesis due to lowered tissue coenzyme A, as was observed in the fat-free groups.

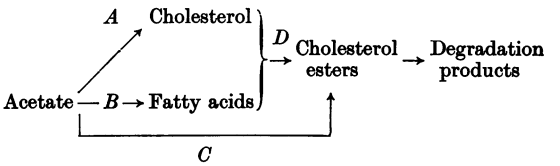
These seemingly anomalous experimental results might be explained as follows. On a fat-free diet the organism synthesizes cholesterol from acetate; thus a two-carbon compound may give rise to a four-carbon compound, such as acetoacetate, and perhaps larger even-numbered carbon compounds, as intermediates in the biosynthesis of the sterol. This process would involve the multiple utilization of acetate to produce intermediates in the biosynthesis which contain even numbers of carbon atoms. Each addition of acetate to the preceding intermediate, for instance acetate plus acetate yielding acetoacetate, would then involve coenzyme A, and at the same time produce an intermediate common to cholesterol biosynthesis and fatty acid catabolism. Thus if the tissue coenzyme-A concentration is reduced, the ability to synthesize acetoacetate, or other even-numbered carbon atom compounds, from acetate will be reduced, and if these compounds are intermediates in cholesterol biosynthesis, then this theory would explain the drop in ester cholesterol observed in the fat deficient, pantothenate-deficient group.

If neutral fat is present in the diet, then the catabolism of the fatty acids could produce C_4, C_6, and higher even-numbered carbon atom intermediates, which may be required in the biosynthesis of cholesterol. This process would in all probability reduce the tissue coenzyme-A requirement to produce a given amount of a certain intermediate. Hence the presence of fatty acids in the diet would overcome the partial inhibition of the synthesis of these intermediates due to the decreased tissue coenzyme-A content.

On this theory, based on these experimental facts, it seems possible that certain even-numbered fatty acids, or fatty acid derivatives (other than acetate), can enter the cholesterol biosynthetic pathway without prior degradation to acetate. The simplest example of this mechanism is the direct incorporation of acetoacetate into cholesterol without previous degradation to acetate, which has been shown *in vitro* by Brady & Gurin (1951).

Bloch (1951) has described experiments in which cholesterol and mixed non-volatile fatty acids have been isolated after the administration of ^{14}C-labelled acetate. Since the specific radioactivity of the former was higher than the 'specific radioactivity' of the latter, Bloch concluded that cholesterol could not be synthesized via the non-volatile fatty acids. While this conclusion may be correct, the use of data derived from the so-called 'specific radioactivity of a mixture' cannot be justified in support of this theory.

Finally, it could be argued that decreased ester cholesterol in the presence of decreased coenzyme-A concentration could be due to an interference with the cholesterol esterification reaction. The following argument would appear to dismiss this hypothesis.



1. Cholesterol is synthesized from acetate (reaction *A*).
2. Fatty acids are synthesized from acetate (reaction *B*).
3. Ester cholesterol is synthesized from cholesterol plus fatty acids (reaction *D*).

Hence ester cholesterol is synthesized from acetate, and this work suggests that the biosynthesis of ester cholesterol is dependent on coenzyme A.

If the reduction in ester cholesterol due to decreased coenzyme A were mediated through reaction *D*, and the biosynthesis of cholesterol proceeded normally when the coenzyme-A level was lower than usual, then there should be a corresponding increase in free cholesterol. This was not observed.

If the reaction *D* were the point at which the blockage in ester cholesterol biosynthesis occurred due to decreased coenzyme A, then overcoming this inhibition by fatty acids (presumably by a 'mass action effect') as observed by the author should result in an increase in ester cholesterol at the expense of the free cholesterol. This was not observed.

Hence it can be deduced that reaction *A*, the synthesis of cholesterol from acetate appears to be coenzyme-A dependent.

SUMMARY

1. It has been observed that if rats are maintained on a fat-free diet, the liver ester cholesterol concentration and the plasma ester cholesterol concentration appear to be dependent on dietary pantothenate and hence on the coenzyme-A content of the tissues.

2. The presence of neutral fat in the diet can annul these effects.

3. A possible explanation of these results is submitted.

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