ENZYMATIC CONVERSION OF SQUALENE TO CHOLESTEROL BY AN ACETONE POWDER OF RAT LIVER MICROSOMES*

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Received October 10, 1967

The in vitro biosynthesis of cholesterol from acetate was first demonstrated by Bucher (1953) using a cell-free supernatant of rat liver prepared by centrifugation at 500 x g. Subsequent studies (Bucher and McGarrahan, 1956; Tchen and Bloch, 1957; Olson et al., 1957) have shown that the enzymes responsible for the conversion of squalene to cholesterol reside in the particulate fraction sedimented at 105,000 x g (microsomes) and that the 105,000 x g supernatant of rat liver stimulates cholesterol formation.

Unfortunately, attempts to purify the microsomal fraction with reference to this biosynthetic sequence have been generally unsuccessful or inconclusive (Tchen and Bloch, 1957; Olson et al., 1957; Bloch, 1965), with the one exception of the enzyme responsible for the cyclization of 2, 3-oxidosqualene (Dean et al., 1967). To the best of our knowledge the preparation of an acetone powder capable of the enzymatic conversion of squalene to cholesterol has not been previously reported. This report describes the successful preparation of an acetone powder from rat liver microsomes which,

*This investigation was supported by U.S.P.H.S. Grant AM-10628 and by National Multiple Sclerosis Society Grant No. 442; presented in part at the September, 1967 meeting of the New Mexico Academy of Science, Albuquerque, New Mexico, The New Mexico Academy of Science Bulletin, No. 2, 8, 23 (1967).

when combined with the 105,000 x g supernatant of rat liver (which alone is inert), is capable of sterol biosynthesis in high yield.

Materials and Methods. Male Sprague-Dawley rats, weighing approximately 200 g, were used. Homogenates were prepared by a method similar to that of Frantz and Bucher (1954) except that (a) a tight fitting pestle was used and (b) the buffer employed was a 0.02 M phosphate buffer, pH 7.0, containing 1×10^{-4} M EDTA. Centrifugation at 1000 x g for 15 minutes removed cell nuclei and unbroken cells; the 1000 x g supernatant was centrifuged at 20,000 x g for 10 minutes to yield the 20,000 x g supernatant which was used in the incubation shown in Figure 1; the 20,000 x g supernatant was centrifuged at 105,000 x g for 60-90 minutes yielding a microsomal pellet which was resuspended in buffer to prepare the acetone powder. The 105,000 x g supernatant was centrifuged for an additional 60 minutes and the upper half (after discarding the fatty layer) utilized in the incubations.

The acetone powder was prepared at -10 to -15° C by a method similar to that described by Morton (1955) followed by acetone and diethyl ether washes and final drying under a stream of nitrogen. The powder was then resuspended in buffer and used immediately.

Incubations were carried out after the addition of cofactors (see Figures 1 and 2) at 37° C in an atmosphere of oxygen for two hours in a Dubnoff Shaker. The reaction was stopped by the addition of alcoholic-KOH and the incubation mixtures were extracted in a manner similar to that previously described (Scallen et al., 1962) except that refluxing was omitted. The petroleum ether extract was reduced to dryness by a stream of nitrogen, and the residue was dissolved in benzene and applied to a 1.2 x 100 cm silicic acid column (Frantz, 1963).

Tritium labeled squalene (squalene-^{3}H) was prepared biosynthetically from DL-mevalonic acid lactone-2-^{3}H (purchased from Nuclear Chicago) by

anerobic incubation with the 20,000 x g supernatant of rat liver in a manner similar to that described by Tchen (1963), except that purification was achieved using 5:1 Unisil[1] silicic acid:supercel columns with 9:1 carbon tetrachloride:benzene as eluting solvent. Squalene-^{3}H was further characterized via the thiourea adduct (Goodman and Popjack, 1960); specific activity was at least 10,000 cpm/μg (counting efficiency 30%) based upon calculations similar to those presented by Rothblat et al. (1962). Squalene-^{3}H was dissolved in a 2:1 solution of dioxane:propylene glycol and added to the incubations in volumes of 5-25 μl.

Results. Figure 1 shows the silicic acid chromatogram obtained after incubation of a 20,000 x g supernatant of rat liver with squalene-^{3}H. Figure 2 is a similar experiment done instead with an acetone powder of rat liver microsomes supplemented with the 105,000 x g supernatant of rat liver. In both cases, significant formation of radioactive cholesterol occurred. This conclusion is supported by two observations. First, as can be seen in the figures, tubes 44-53 (Figure 1) and tubes 45-55 (Figure 2), which contain carrier cholesterol added prior to chromatography, also contain a peak of radioactivity which is closely coincident with cholesterol measured colorimetrically. Second, further proof that the radioactivity chromatographically identical with carrier cholesterol was indeed radioactive cholesterol synthesized by the respective preparation was obtained by passage thru the dibromide (Fieser, 1953; Frantz et al., 1959). Specific activity obtained before the dibromide (Figure 1) was 8.7 cpm/mg and after the dibromide 8.6 cpm/mg; for the acetone powder (Figure 2) specific activity obtained before the dibromide was 3.2 cpm/mg and after the dibromide 3.2 cpm/mg.

Significantly, a control incubation utilizing the 105,000 x g supernatant of rat liver, the same cofactors as shown in the Figures and

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[1]Clarkson Chemical Company, Williamsport, Pa.

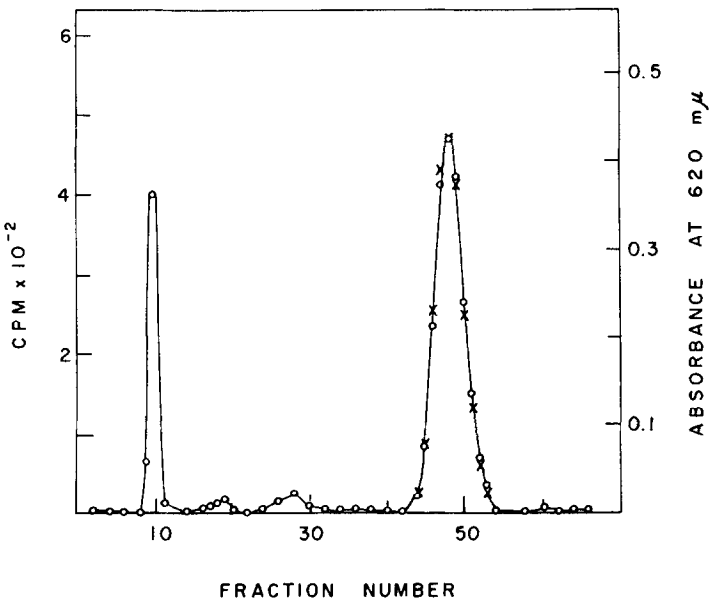

Figure 1. Distribution of radioactivity on a silicic acid column after a two hour incubation of squalene-^{3}H with a 20,000 x g supernatant of rat liver. The incubation flask contained (a) 20,000 x g supernatant (5 ml), (b) NADPH (5 mg), NAD (2 mg) and nicotinamide (15 mg) dissolved in 1 ml of buffer, and (c) squalene-^{3}H (8000 cpm). Final volume was 6 ml; protein concentration (Lowry et al., 1951), 27 mg/ml. Three milligrams of carrier cholesterol were added prior to chromatography; o——o, cpm; x——x, cholesterol measured colorimetrically (Abell et al., 1952).

squalene-^{3}H showed no incorporation of squalene into sterols, and yielded only unchanged substrate.

Figure 1 shows further that for the 20,000 x g supernatant only two major peaks of radioactivity were detected; Peak I (tubes 9-11) corresponds to squalene, and Peak II (tubes 44-53) is cholesterol. Furthermore, cholesterol accounted for approximately 62% of the recovered radioactivity.

In contrast, the acetone powder showed the presence of two additional radioactive peaks (tubes 17-22-Peak II and tubes 25-34-Peak III). These peaks have not been characterized, however their chromatographic mobility suggests that they may represent lanosterol and/or steroids very similar in structure to lanosterol. In addition, it was noted that acetone powders prepared with buffer containing 0.01 M mercaptoethanol accumulate Peaks II

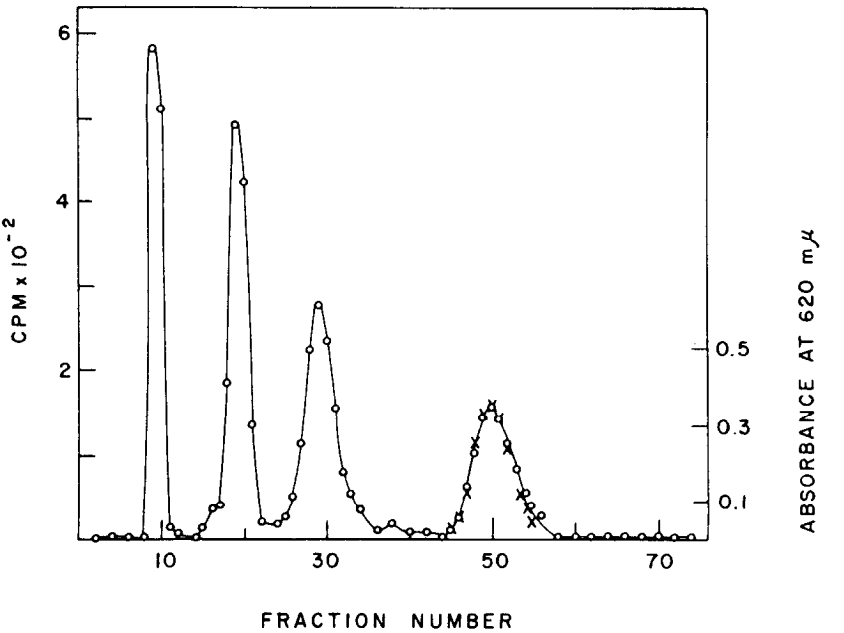

Figure 2. Distribution of radioactivity on a silicic acid column after a two hour incubation of squalene-^{3}H with an acetone powder of rat liver microsomes. The incubation flask contained (a) acetone powder resuspended in 0.02 M phosphate buffer (see Methods, 5 ml), (b) 105,000 x g supernatant of rat liver (5 ml), (c) NADPH (5 mg), NAD (2 mg) and nicotinamide (15 mg) dissolved in 1 ml of buffer, and (d) squalene-^{3}H (8000 cpm). Final volume was 11 ml; protein concentration, 21 mg/ml. Three milligrams of carrier cholesterol were added prior to chromatography; o——o, cpm; x——x, cholesterol measured colorimetrically.

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and III (Figure 2), but no cholesterol formation could be detected. In Figure 2 cholesterol accounted for 20% of the recovered radioactivity, and sterol products (sum of Peaks II, III and IV) accounted for 75%-80% of the recovered radioactivity.

Discussion. We have demonstrated for the first time the conversion of squalene-^{3}H to cholesterol by an acetone powder preparation of rat liver microsomes. This is important because previous attempts to prepare acetone powders which would carry out these reactions were unsuccessful (Tchen and Bloch, 1957).

Also of significance is the high yield of sterol products synthesized, 62% for the 20,000 x g supernatant and 75-80% for the acetone powder pre-

paration. These figures are considerably higher than those previously reported (Tchen and Bloch, 1957; Tchen, 1962) which showed conversions of 20-25%.

It has been proposed (Bloch, 1965) that the enzymes involved in the conversion of squalene to cholesterol might be lipoprotein in nature with the lipid essential for catalytic activity. It is of interest that in our experiments considerable amounts of lipid material were removed by the acetone and diethyl ether procedures employed to prepare the powder. Thus, significant amounts of lipid material can be removed without abolition of enzymatic activity. In addition, evaporation of the acetone and diethyl ether extracts and recombination of this lipid material with the acetone powder was without effect. However, bound phospholipids would not necessarily be removed from the microsomes by the solvents employed here, so their possible essentiality for these enzymatic reactions cannot be excluded by our experiments.

Our data as well as that presented recently by another laboratory (Dean et al., 1967) suggests an alternative hypothesis, namely, that the lipid associated with the microsomal fraction may not be required for enzymatic activity in sterol biosynthesis but rather may serve a structural function in the endoplasmic reticulum.

The second hypothesis is under active investigation in our laboratory in studies aimed at obtaining these enzymes in water soluble form.

Summary. The first successful preparation of an acetone powder, prepared from rat liver microsomes and supplemented with the 105,000 x g supernatant of rat liver, capable of converting squalene to cholesterol has been demonstrated. Cholesterol accounted for 20% of the recovered radioactivity. The possibility that the lipid removed by acetone and ether extraction may not be essential for enzymatic activity in these reactions is discussed.

References

Abell, L. L., Levy, B. B., Brody, B. B., and Kendall, F., J. Biol. Chem., 195, 357 (1952).

Bloch, K., Science, 150, 19 (1965).
Bucher, N. L. R., J. Am. Chem. Soc., 75, 496 (1953).
Bucher, N. L. R., and McGarrahan, K., J. Biol. Chem., 222, 1 (1956).
Dean, P. D. G., Ortiz De Montellano, P. R., Bloch, K., and Corey, E. J., J. Biol. Chem., 242, 3014 (1967).
Fieser, L. F., J. Am. Chem. Soc., 75, 5421 (1953).
Frantz, I. D., Jr., and Bucher, N. L. R., J. Biol. Chem., 206, 471 (1954).
Frantz, I. D., Jr., Davidson, A. G., Dulit, E., and Mobberley, M. L., J. Biol. Chem., 234, 2290 (1959).
Frantz, I. D., Jr., J. Lipid Res., 4, 176 (1963).
Goodman, De W. S., and Popjak, G., J. Lipid Res., 1, 286 (1960).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
Morton, R. K., in "Methods in Enzymology" Edited by Colowick, S. P. and Kaplan, N. O., Academic Press, New York, 1955. Vol. I, p. 34.
Olson, J. A., Jr., Lindberg, M., and Bloch, K., J. Biol. Chem., 226, 941 (1957).
Rothblat, G. H., Martak, D. S., and Kritchevsky, D., Anal. Biochem., 4, 52 (1962).
Scallen, T. J., Condie, R. M., and Schroepfer, G. J., Jr., J. Neurochem., 9, 99 (1962).
Tchen, T. T., and Bloch, K., J. Biol. Chem., 226, 921 (1957).
Tchen, T. T., in "Methods in Enzymology" Edited by Colowick, S. P. and Kaplan, N. O., Academic Press, New York, 1962, Vol. V, p. 494.
Tchen, T. T., in "Methods in Enzymology" Edited by Colowick, S. P. and Kaplan, N. O., Academic Press, New York, 1963, Vol. VI, pp. 509-11.