Utilization of [5-^{3}H]Mevalonate for Sterol and Steroid Synthesis by the Guinea Pig Adrenal *in Vitro* *

R. B. Billiar,† A. Oriol-Bosch,‡ and K. B. Eik-Nes

ABSTRACT: Guinea pig adrenal slices were incubated for 8 hours in Krebs-Ringer phosphate buffer containing 200 mg % glucose in the presence of a total of approximately 1.24 mc DL-[5-^{3}H]mevalonate. Tritium was incorporated into free and esterified sterol by the adrenal tissue. [^{3}H]Corticosterone and 11-[^{3}H]deoxycorticosteroids, isolated from the incubation media, were crystallized to constant specific activity.

Acetate is the 2-carbon precursor unit for cholesterol formation in the liver (Popjak and Cornforth, 1960), and the isoprenoid scheme formulated by Bonner and Arreguin (1949) applies to the transformation of acetate to cholesterol in this organ (Popjak and Cornforth, 1960). A key intermediate (Tavormina *et al.*, 1956) in this pathway is mevalonic acid.

Rabinowitz and Ragland (1958) claimed that [^{14}C]-mevalonic acid was incorporated into estradiol in a homogenate from a testicular tumor. Normal adrenal, testicular, and *corpora luteal* tissues will convert [1-^{14}C]acetate to radioactive steroids but fail to utilize added [^{14}C]mevalonic acid as substrate for such biotransformations (Bryson and Sweat, 1962; Savard *et al.*, 1960; Hall and Eik-Nes, 1962; Mason *et al.*, 1962). The pathway(s) for cholesterol biosynthesis in steroid-forming organs has not been studied in great detail but Salokangas *et al.* (1964) have recently demonstrated that rat testicular homogenate can convert [^{14}C]isopentenyl pyrophosphate to squalene, lanosterol, and cholesterol. Thus the endocrine tissues may utilize a similar pathway as do mammalian liver and yeast for the conversion of acetate to cholesterol.

We have recently reported an *in vitro* adrenal system which will efficiently label the adrenal sterol pools from [^{14}C]acetate (Billiar and Eik-Nes, 1964). Such a system was used to investigate the role of mevalonic acid in adrenal steroid production.

Materials and Methods

Guinea pigs (300–500 g) of the Hartley strain or utility breed were decapitated; the adrenals were removed, cleaned of adhering tissues, weighed, and cut into approximately 0.7-mm-thick slices. The slices were placed in incubation flasks containing 10 ml 200 mg % glucose in Krebs-Ringer buffer, *p*H 7.4 (Umbreit *et al.*, 1957), and the flasks were chilled while the desired amount of slices was accumulated. About 4 minutes elapsed from the removal of the animal from its cage to the transfer of its sliced adrenal into the incubation flask. When between 400 and 500 mg adrenal tissue was present in each flask, [1-^{14}C]acetate or [5-^{3}H]-mevalonate was added, the flasks were oxygenated for 3 minutes with 95% O_2–5% CO_2, tightly stoppered, and incubated for 2 hours at 37° with agitation. The medium was then removed and discarded, the tissue was washed with the buffer, and the washes were discarded. Fresh medium containing radioactive substrate (Table I) was added, and the incubation flasks were oxygenated and incubated for another 2 hours. This latter procedure was repeated until the slices had been incubated for a total of 8 hours. At the initiation of the last 2-hour incubation, 3 USP units ACTH/100 mg slices was added to all incubation flasks.[1]

After the final incubation period the medium of each flask was quantitatively transferred to extraction tubes and the slices were rinsed several times. The medium and rinses were combined and extracted three times with twice their volume of ethyl acetate. The extracts were combined, reduced to 10 ml in a nitrogen atmosphere at 45°, and washed first with 1 ml of 0.1 N aqueous sodium hydroxide and then with 0.1 volume of deionized water. The washed extracts were then evaporated to dryness in nitrogen, and radioactive steroids were isolated and identified in these residues. The washed tissue was homogenized in about 10 ml of an ethanol-acetone-ether (4:4:1, v/v/v) mixture, and this homogenate was used for sterol determinations (Kabara *et al.*, 1961).

Isolation Procedures: Steroids. The extracts of the incubation medium were chromatographed in various

* From the Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City. *Received August 24, 1964.* This investigation was aided by grants (T1 GM 152-05 and T4 CA 5000) from the U.S. Public Health Service.

† Portions of this communication were abstracted from a dissertation submitted by R. B. Billiar to the graduate school of the University of Utah in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Department of Biological Chemistry, Harvard University Medical School, Boston, Mass.

‡ Present address: Department of Physiology, University of Madrid, Madrid, Spain.

[1] Abbreviation used in this work: ACTH, adrenocorticotropic hormone.

TABLE I: Incorporation of ^{14}C from [1-^{14}C]Acetate and of ^{3}H from DL-[5-^{3}H]Mevalonate into Free and Esterified Adrenal Tissue Sterols.[a]

Flask	Addition: Acetate	Addition: Mevalonate	Incorporation: ^{14}C (dpm/μM)	^{14}C (dpm in fraction)	^{14}C (% conversion)	^{3}H (dpm/μM)	^{3}H (dpm in fraction)	^{3}H (% conversion)
					Free Sterols			
1	50 μc each 2nd hour	313 μc each 2nd hour	99×10^3	363×10^3	0.83×10^{-1}	417×10^3	1.53×10^6	0.56×10^{-1}
2	50 μc each 2nd hour except for last 2-hour period	As above	66×10^3	292×10^3	0.71×10^{-1}	329×10^3	1.55×10^6	0.56×10^{-1}
3	50 μc only for last 2 hours	As above	31×10^3	137×10^3	1.25×10^{-1}	306×10^3	1.35×10^6	0.49×10^{-1}
4	None	As above				204×10^3	837×10^3	0.30×10^{-1}
					Esterified Sterols			
1			35×10^3	598×10^3	1.45×10^{-1}	155×10^3	2.68×10^6	0.97×10^{-1}
2			22×10^3	388×10^3	1.17×10^{-1}	110×10^3	1.92×10^6	0.70×10^{-1}
3			6.3×10^3	111×10^3	1.01×10^{-1}	95×10^3	1.68×10^6	0.61×10^{-1}
4						63×10^3	1.09×10^6	0.40×10^{-1}

[a] All incubations were done for a total of 8 hours. Guinea pig adrenal slices (*ca.* 400 mg) were incubated for four consecutive 2-hour periods in 200 mg % glucose–Krebs-Ringer phosphate buffer (*p*H 7.4). Fresh incubation medium was added at the initiation of each 2-hour incubation and the tissues were oxygenated with 95% O_2–5% CO_2 and incubated at 37° with agitation. [1-^{14}C]Acetate and [5-^{3}H]mevalonate were added to the medium as indicated in this table and ACTH (3 USP units/100 mg adrenal tissue) was added at the initiation of the last incubation period to all flasks.

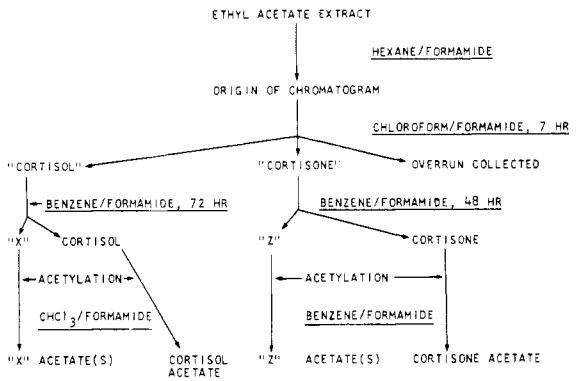

FIGURE 1: Flow sheet indicating steps of purification for incubation medium cortisol and cortisone.

paper partition chromatographic systems (Burton *et al.*, 1951; Zaffaroni and Burton, 1951), and initial chromatography was done in the solvent system hexane-formamide (Figure 1). Localization of ultraviolet absorbing compounds on the chromatograms and acetylation of the steroids were done as described by our laboratory (Oriol-Bosch and Eik-Nes, 1964). Figures 1 and 2 outline the sequence of paper chromatographic systems used to isolate adrenal steroids. It should be noted that compounds "X" and "Z" of Figure 1 and compound "R" of Figure 2 are unknowns.

Radioactive areas were located with a scanning window on a windowless Geiger counter (Berliner *et al.*, 1957). Final quantification of radioactivity was done with a Packard Tri-Carb liquid scintillation spectrophotometer, dissolving the sample in 10 ml of scintillation fluid (4 g diphenyloxazole and 50 mg 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 liter toluene). When carbon-14 and tritium were present in the same sample, the simultaneous equation formula of Okita *et al.* (1957) was used to calculate the quantity of ^{14}C and ^{3}H. When crystallizing a biosynthesized radioactive compound to constant specific activity, we weighed a portion of the crystals on a Cahn electrobalance and estimated radioactivity in the crystals by liquid scintillation spectrophotometry.

Isolation Procedures: Sterols. The tomatine method of Kabara *et al.* (1961) was used to isolate free and esterified sterols in the adrenal slices. Sterol mass was determined by the Liebermann-Burchard color reaction (Kabara *et al.*, 1961) within 35 minutes after the addition of the freshly prepared reagent. The radioactivity in the sterol fractions was estimated as described (Kabara *et al.*, 1961) and was counted within a day of sample preparation.

Sodium [1-^{14}C]acetate with a specific activity of about 50 μc/μmole was purchased from New England Nuclear Corp. The *N,N*-dibenzylethylenediamine salt of DL-[5-^{3}H]mevalonic acid was also purchased from New England Nuclear Corp. and had a stated specific activity of 206 μc/μmole. The mevalonic acid was converted to its sodium salt by the method of Hoffman *et al.* (1957) before being used in the incubations. ACTH was a gift of Drs. M. Glenn of the Upjohn Co. and J. Fisher of Armour Research Laboratories.

Results

Both ^{14}C from [1-^{14}C]acetate and ^{3}H from [5-^{3}H]-mevalonate were incorporated into free and esterified adrenal sterols (Table I). When decreasing concentrations of [1-^{14}C]acetate were employed and the concentration of [5-^{3}H]mevalonate was kept constant, the $^{3}H/^{14}C$ ratio in the sterols increased. As observed previously (Billiar and Eik-Nes, 1964), [1-^{14}C]acetate was still converted to [^{14}C]sterol by adrenal slices which had been incubated for 6 hours before being exposed to this substrate (flask 3, Table I).

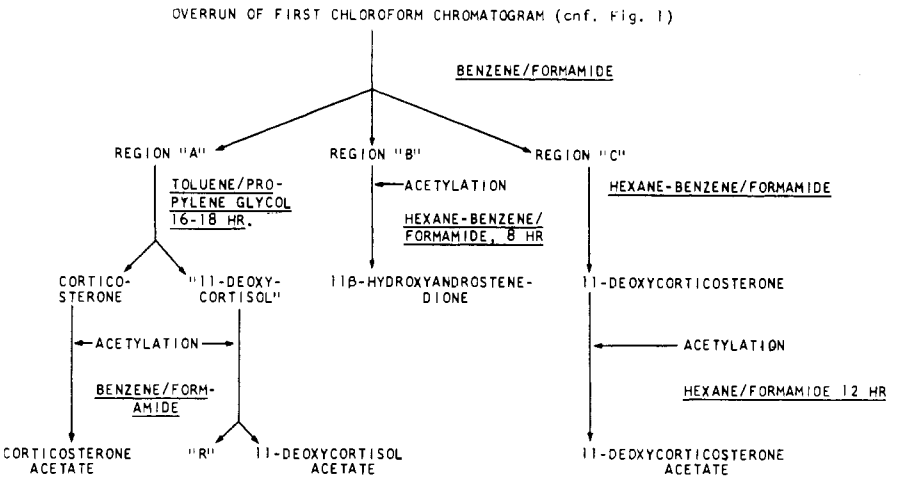

FIGURE 2: Flow sheet indicating steps of purification for incubation medium corticosterone, 11-deoxycortisol, 11β-hydroxyandrostenedione, and 11-deoxycorticosterone. These compounds were present in the overrun from the original hexane/formamide chromatogram, cf. Figure 1.

TABLE II: Incorporation of ^{3}H from [5-^{3}H]Mevalonate into Free and Esterified Adrenal Tissue Sterols.[a]

Flask No.	Free Sterols (dpm/μM)	Free Sterols (dpm in fraction)	Free Sterols (% conversion)	Esterified Sterols (dpm/μM)	Esterified Sterols (dpm in fraction)	Esterified Sterols (% conversion)
1	50×10^{3}	187×10^{3}	0.71×10^{-2}	9×10^{3}	22×10^{3}	0.84×10^{-3}
2	64×10^{3}	171×10^{3}	0.65×10^{-2}	8×10^{3}	22×10^{3}	0.84×10^{-3}
3	34×10^{3}	140×10^{3}	0.53×10^{-2}	7×10^{3}	20×10^{3}	0.76×10^{-3}
4	47×10^{3}	187×10^{3}	0.71×10^{-2}	10×10^{3}	23×10^{3}	0.87×10^{-3}

[a] All incubations were done for a total of 8 hours. The same incubation procedure was employed as described in the text of Table I except no [1-^{14}C]acetate was used. Between 510 and 540 mg fresh weight of adrenal slices were present in each of the four flasks. The incubation media from the final 2-hour incubation (6–8 hours) were combined and used for steroid analysis, and the slices from each flask were analyzed separately for tomatine-precipitable sterols.

TABLE III: Crystallization of [^{3}H]Corticosterone Acetate Isolated from Incubation of Guinea Pig Adrenal Slices with [5-^{3}H]Mevalonate.[a]

Crystallization	Solvent	Specific Activity (dpm/mg)
1st	Methanol	250
2nd	Hexane-benzene-methanol (2:2:1)	340
3rd	Ethyl acetate–methanol (1:1)	320
4th	Ethanol	340

[a] The [^{3}H]corticosterone acetate was isolated from the experiment described in the text of Table II. Unlabeled authentic corticosterone acetate was added at the initiation of the crystallization procedure.

TABLE IV: Crystallization of 11-[^{3}H]Deoxycorticosterone Acetate Isolated from Incubations of Guinea Pig Adrenal Slices with [5-^{3}H]Mevalonate.[a]

Crystallization	Solvent	Specific Activity (dpm/mg)
1st	Methanol	210
2nd	Hexane-benzene (2:1)	240
3rd	Ethyl acetate–methanol (1:1)	260
4th	Ethanol	240

[a] The 11-[^{3}H]deoxycorticosterone acetate was isolated from the experiment described in the text of Table II. Unlabeled authentic 11-deoxycorticosterone acetate was added at the initiation of the crystallization procedure.

In the second series of incubations only [5-^{3}H]-mevalonate was incubated with adrenal slices. Adrenal tissue sterols (Table II) were separated and the steroids in the incubation medium were isolated and identified. The purified adrenal steroids were crystallized in several different solvent combinations. Although [^{3}H]cortisol and 11-[^{3}H]deoxycortisol could not be crystallized to constant specific activity, the crystallization data for [^{3}H]corticosterone (Table III), 11-[^{3}H]deoxycorticosterone (Table IV), and 11β-[^{3}H]hydroxyandrostenedione (Table V) indicate that tritium from [5-^{3}H]mevalonate was incorporated into these compounds.

Discussion

5-D-Mevalonic acid was used by Rilling and Bloch (1959) investigating squalene biosynthesis. Popjak *et al.* (1961) demonstrated that 11 of the possible 12 deuterium atoms of 5-D-mevalonic acid were incorporated into squalene. Squalene is a precursor of cholesterol in the liver (Langdon and Bloch, 1953), and the data of Caspi *et al.* (1962) indicate that the carbon of acetate has the same distribution in the cholesterol molecules synthesized by the adrenal as it does in the cholesterol molecule synthesized by the liver. It is, therefore, probable that in our investigation the ^{14}C of acetate and ^{3}H from [5-^{3}H]mevalonic acid follow similar pathways for sterol biosynthesis in the adrenal gland. Since these sterols served as precursors for the corticosteroids (Werbin and Chaikoff, 1961; Krum *et al.*, 1964), it was not surprising that the adrenal sterol pool labeled with ^{3}H from [^{3}H]mevalonic acid could be utilized for the production of ^{3}H-containing adrenal steroids (Tables III–V).

Nevertheless, ^{3}H from [5-^{3}H]mevalonic acid may have a different metabolic fate than that suggested if carbon-5

TABLE V: Crystallization of 11β-[^{3}H]Hydroxyandrostenedione Isolated from Incubation of Guinea Pig Adrenal Slices with [5-^{3}H]Mevalonate.[a]

Crystallization	Solvent	Specific Activity (dpm/mg)
1st	Methanol	270
2nd	Hexane-benzene (1:1)	230
3rd	Toluene	180
4th	Ethanol	280

[a] The 11β-[^{3}H]hydroxyandrostenedione was isolated from the experiment described in the text of Table II. Unlabeled authentic 11β-hydroxyandrostenedione was added at the initiation of the crystallization procedure.

of mevalonic acid were to undergo oxidation and were transferred to reduce pyridine nucleotide. According to Popjak and Cornforth (1960), "a mevalonic acid molecule seems to have substantially no metabolic future except as a source of isoprenoid unit." Hydroxymethylglutaryl-CoA conversion to mevalonic acid appears to be essentially irreversible (Lynen, 1959; Conn *et al.*, 1959). Ogilvie and Langdon (1959) observed a small conversion of mevalonic acid to olefinic acids by a rat liver supernatant system, but the presence of microsomes in the system abolished this reaction. Furthermore, Tavormina *et al.* (1956) demonstrated an essentially quantitative conversion of mevalonic acid to sterol in a rat liver cell-free system. Therefore, the reintroduction of lost ^{3}H atoms from [5-^{3}H]mevalonic acid into intermediates between acetate and cholesterol seems improbable.

Previous experience indicated that adrenal slices incubated for prolonged times maintain biosynthetic capacity (Billiar and Eik-Nes, 1964). Although the present conversion of [^{3}H]mevalonate to [^{3}H]sterols by such slices is relatively small (Tables I and II), it is not known what per cent of the added mevalonate actually entered *into* the cells. Limited access of the substrate to its organized, intracellular conversion site(s) may in part account for earlier failures to observe a conversion of mevalonate to steroid in endocrine tissues. By prolonging the incubation time increased penetration of the substrate to the different metabolic pools may be achieved. This proposed enhancement may be owing in part to altered intra- and/or extracellular membrane permeability. That some adrenal cellular organization still exists in the system employed in these studies is, however, indicated by the fact that slices of guinea pig adrenals incubated for 6 hours will increase production of adrenal steroids when incubated with ACTH for an additional 2 hours (Billiar and Eik-Nes, 1964).

References

Berliner, D. L., Dominguez, O. V., and Westenskow, G. (1957), *Anal. Chem. 29*, 1797.

Billiar, R. B., and Eik-Nes, K. B. (1964), *Federation Proc. 23*, 250.

Bonner, J., and Arreguin, B. (1949), *Arch. Biochem. Biophys. 21*, 109.

Bryson, M. J., and Sweat, M. L. (1962), *Arch. Biochem. Biophys. 96*, 1.

Burton, R. B., Zaffaroni, A., and Keutmann, E. H. (1951), *J. Biol. Chem. 193*, 769.

Caspi, E., Dorfman, R. I., Kahn, B. T., Rosenfeld, G., and Schmid, W. (1962), *J. Biol. Chem. 237*, 2085.

Conn, M. J., Kupieck, F. P., Dekker, E. E., Schlesinger, M. J., and Del Compillo, A. (1959), *Ciba Found. Symp. Biosyn. Terpenes Sterols*, 62.

Hall, P. F., and Eik-Nes, K. B. (1962), *Biochim. Biophys. Acta 63*, 411.

Hoffman, C. H., Wagner, A. F., Wilson, A. N., Walton, E., Shunk, C. H., Wolf, D. E., Holly, F. W., and Folkers, K. (1957), *J. Am. Chem. Soc. 79*, 2316.

Kabara, J. J., McLaughlin, J. T., and Riegel, C. A. (1961), *Anal. Chem. 33*, 305.

Krum, A. A., Morris, M. D., and Bennett, L. L. (1964), *Endocrinology 74*, 543.

Langdon, R. G., and Bloch, K. (1953), *J. Biol. Chem. 200*, 129.

Lynen, F. (1959), *Ciba Found. Symp. Biosyn. Terpenes Sterols*, 95.

Mason, N. R., Marsh, J. M., and Savard, K. (1962), *J. Biol. Chem. 237*, 1801.

Ogilvie, J. W., Jr., and Langdon, R. G. (1959), *J. Am. Chem. Soc. 81*, 754.

Okita, G. T., Kabara, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics 15*, 111.

Oriol-Bosch, A., and Eik-Nes, K. B. (1964), *Metabolism 13*, 319.

Popjak, G., and Cornforth, J. W. (1960), *Advan. Enzymol. 22*, 281.

Popjak, G., Goodman, D. S., Cornforth, J. W., Cornforth, R. H., and Ryhage, R. (1961), *J. Biol. Chem. 236*, 1934.

Rabinowitz, J. L., and Ragland, J. B. (1958), *Federation Proc. 17*, 293.

Rilling, H. C., and Bloch, K. (1959), *J. Biol. Chem. 234*, 1424.

Salokangas, A., Rilling, H. C., and Samuels, L. T. (1964), *Biochemistry 3*, 833.

Savard, K., Goldweber, M., and Goldzieher, J. W. (1960), *Federation Proc. 19*, 169.

Tavormina, P. A., Gibbs, M. H., and Huff, J. W. (1956), *J. Am. Chem. Soc. 78*, 4498.

Umbriet, W. W., Burris, R. H., and Stauffer, J. F. (1957), Manometric Techniques, 3rd ed., Minneapolis, Burgess, p. 149.

Werbin, H., and Chaikoff, I. L. (1961), *Arch. Biochem. Biophys. 93*, 476.

Zaffaroni, A., and Burton, R. B. (1951), *J. Biol. Chem. 193*, 749.