Journal of Chromatography, 190 (1980) 256–262

CHROM. 12,556

Note

Pregnenolone separation from cholesterol using Sephadex LH-20 minicolumns

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(Received October 23rd, 1979)

Cholesterol conversion to pregnenolone is an essential step in the biosynthesis of steroid hormones. This conversion is catalyzed by a mitochondrial monooxygenase system that consists of a flavoprotein (adrenodoxin reductase), an iron sulfur protein (adrenodoxin) and a cytochrome *P*-450 specific for cholesterol side-chain cleavage (SCC) (cytochrome *P*-450$_{scc}$)[1].

The products of the cholesterol SCC reaction, pregnenolone and isocapraldehyde, have been assayed by a variety of methods which can be grouped under two categories:

(a) Direct assays of pregnenolone: *e.g.*, gas–liquid chromatography (GLC), GLC–mass spectrometry, radioimmunoassay[2–4]. These assays are methods of choice for assaying pregnenolone under conditions where there is considerable endogenous cholesterol or for metabolism of cholesterol derivatives that are not commercially available with a radiolabel.

(b) Radiometric assays of pregnenolone or isocapraldehyde: This group of assays require a procedure for the separation of the radiolabeled substrate and product, *e.g.*, thin-layer chromatography (TLC)[5,6]. The simplest assay of this group does not require extraction of the incubation media[7]. It is based on elution of the side chain fragment, isocapraldehyde, through an alumina column which adsorbs cholesterol in the presence of a buffer.

The very low solubility of cholesterol in aqueous solutions[8] presents a problem in the assay of purified preparations of cytochrome *P*-450$_{scc}$. The inclusion of 0.3% Tween 20 in assay buffers increases the solubility of cholesterol, and at least partly as a result of this, the activity of purified cytochrome *P*-450$_{scc}$ increases from 1–2 to 16–18 nmol pregnenolone produced per min per nmol cytochrome *P*-450$_{scc}$ (ref. 6). In our preliminary experiments to determine the turnover number of purified cytochrome *P*-450$_{scc}$, we attempted to use the alumina column method[7] with Tween 20 containing buffers. However, we noted that the detergent significantly prevents the adsorption of cholesterol on the alumina column.

Here, we report a new procedure for the separation of radiolabeled pregnenolone and cholesterol using minicolumns of Sephadex LH-20 and a simple solvent system. Additionally, we describe a Sephadex LH-20 procedure for the purification of radiolabeled cholesterol.

MATERIALS AND METHODS

Chemicals

Sephadex LH-20, NADPH (Type I; Sigma, St. Louis, Mo., U.S.A.); [1α,2α(n)-^{3}H]cholesterol (43 Ci/mmol), [4-^{14}C]pregnenolone (55 mCi/mmol; Amersham, Arlington Height, Ill., U.S.A.); and methanol (Fisher, Pittsburgh, Pa., U.S.A.) were purchased from the indicated sources. After purification by the procedure described below, [^{3}H]cholesterol was diluted with carrier cholesterol to a specific activity of 7 to 9 μCi/μmol at a concentration of 16 m*M* in N,N-dimethyl formamide (DMF).

Enzymes

Adrenodoxin, adrenodoxin reductase and cytochrome *P*-450$_{scc}$ were purified by modifications of published procedures[6,9,10] yielding enzymes with the same purity as those reported.

Incubations

Incubations are performed in 5-ml polypropylene tubes (Fisher) and all pipettings are carried out with polypropylene tips. A volume (133 μl × number of incubations) of 25 m*M* potassium phosphate (KP) buffer, pH 7.2, containing 0.45% Tween 20 is heated to 70° and maintained at that temperature in a water bath for 2 min. [^{3}H]Cholesterol, 16 m*M* in DMF, (2.5 μl/133 μl buffer) is added to the solution which is kept at 70° for an additional 5 min with continuous shaking. The solution is then transferred to a 37° water bath and is maintained at this temperature for the duration of the experiment. Duplicate 20-μl aliquots are placed in scintillation vials to determine the total amount of ^{3}H in solution. Aliquots (133 μl) are transferred to individual incubation tubes. Each incubation solution is adjusted to 180 μl with 25 m*M* KP buffer without detergent. Enzymes, 5 μl 180 μ*M* adrenodoxin, 5 μl 12 μ*M* adrenodoxin reductase and 5 μl 10.8 μ*M* cytochrome *P*-450$_{scc}$ are added in that order to give a final concentration of 4.5, 0.3 and 0.27 μ*M*, respectively in 200 μl final incubation volume. The reaction is initiated by the addition of 5 μl 40 m*M* NADPH in KP to give a final concentration of 1 m*M*.

The incubations are terminated with 0.2 ml 100% ethanol after 4 min, except in reaction time course studies. As a recovery standard [^{14}C]pregnenolone (1600 cpm) is added in 15 μl benzene and the solution mixed by vortex for 15 sec.

Steroid extractions

After terminating the reaction, add 2 ml methylene chloride and vortex for 15 sec. Withdraw all the aqueous phase and 0.4 to 0.5 ml organic phase with a 1-ml polypropylene pipette tip. As the two phases separate easily and rapidly in the tip, return the lower organic phase into the tube and discard the aqueous phase. Dry down under nitrogen and dissolve sample in 0.2 ml 40% methanol.

Sephadex LH-20 systems for [^{3}H]cholesterol purification and [^{3}H]pregnenolone separation

General comments. (a) In order not to disturb the settled gel, the solvents should be pipetted slowly into the column. (b) Sample applications onto the gel preferably should be done with polypropylene pipette tips because of their non-

wetting characteristic. (c) Each volume of solvent applied onto the settled gel should be allowed to drain completely before the next volume is added. (d) After sample application the eluting solvents should be applied as soon as the previous solvent drains completely; otherwise, the steroids may diffuse through the gel or the column may dry out.

[^{3}H]Cholesterol purification. Chromatography is carried out using a glass column (0.7 × 12 cm) with a 20-ml reservoir (Erway Lab. Glassware, Oregon, Wisc., U.S.A.). The following procedure is used: swell 1.1 g Sephadex LH-20 in 9 ml packing solvent (benzene–methanol, 1:1), for at least 4 h. Stir the gel with a glass rod and pour the suspension into the column. Rinse twice with 5 to 7 ml packing solvent and empty into the column. As the solvent flows, the gel bed should reach a height of 12 cm. Fill the reservoir with 20 ml eluting solvent (isooctane–benzene–methanol, 90:5:5). As the solvent drains, the gel bed should shrink to about 9 cm. Dissolve previously dried down sample of radioactive cholesterol in 0.2 ml eluting solvent and apply onto the gel bed with a 0.2 ml rinse. Pipette 3 ml eluting solvent onto the column. Dry down the 3.4 ml of solvent which elutes from the column and which contains purified radioactive cholesterol under a nitrogen stream and dissolve in benzene for future use. Check the purity of an aliquot of the radioactive cholesterol by TLC on silica gel [benzene–ethyl acetate–acetone, 6:1:1 (ref. 5) or cyclohexane–ethyl acetate, 6:4 (ref. 6)] and compare the percentage of radioactivity co-migrating with carrier cholesterol.

Pregnenolone separation from cholesterol. Chromatography is carried out using polypropylene columns (0.7 cm × 4 cm) with a 10-ml reservoir and a polyethylene gel bed support (Bio-Rad). The procedure consists of the following steps: (1) swell Sephadex LH-20 (0.4 g/column) in 100% methanol overnight and adjust the volume of the solvent phase to equal the gel phase. (2) Completely mix up the gel in methanol and pipette with a Pasteur pipette 3 ml of the suspension into the column. Fill the reservoir with 100% methanol. (3) As methanol drains, if necessary, adjust the gel bed height to 4 cm by adding or withdrawing gel suspension with the aid of a Pasteur pipette. Tap the column to achieve a level gel surface. (4) Apply the sample in 0.2 ml 40% methanol and rinse with an additional 0.2 ml 40% methanol. (5) Pipette 3 ml 50% methanol into the column. (6) Discard the 3.4 ml collected after sample application. (7) Pipette 3 ml 50% methanol onto the column and collect the effluent in 5-ml soda lime glass vials with polyethylene stopper having double sealing rings (Research Products International). (8) Dry the effluent on a heating block maintained at no more than 100°. Add 3 ml liquid scintillation counting solution (Organic Scintillant, Amersham) into the dry vial and determine ^{3}H and ^{14}C radioactivity (see below).

Ten to fifteen columns can be conveniently run simultaneously. Columns may be set and be ready to use within 1 h. For ten samples, step 7 is completed within 45 min after sample application.

The columns can be reused indefinitely. One day exposure to 100% methanol does not damage polypropylene or polyethylene. Sephadex LH-20 can also be used at least five times. To reuse the gel and the column, first elute [^{3}H]cholesterol with 5 ml methanol collected at step 3 and discard. Then transfer the gel into a beaker and wash with methanol until no radioactivity is detected in the solvent.

Radioactivity determination and product quantitation. The radioactivity mea-

surements were performed in a Packard Tricarb liquid scintillation spectrometer. The lower discriminator of the ^{14}C channel is set to reduce the ^{3}H overlap to less than 0.02%. The upper discriminator of the ^{3}H channel is set to reduce ^{14}C overlap to about 15%. Counting efficiency was monitored by automatic external standardization and did not show significant fluctuations (1 to 2%). The amount of [^{3}H]pregnenolone formed in the incubation is calculated by subtracting the incubation blank values (^{3}H levels observed in the [^{3}H]pregnenolone fraction without cytochrome $P\text{-}450_{scc}$ in the incubation) and by correcting for recovery of [^{3}H]pregnenolone as determined by [^{14}C]pregnenolone counts. The ^{3}H counts are converted into pmol on the basis of the specific activity of the added [^{3}H]cholesterol (5 to 7 cpm/pmol).

RESULTS AND DISCUSSION

Purification of radioactive cholesterol

As indicated in the elution profile (Fig. 1), applied cholesterol is recovered quantitatively in the first 4 ml, while pregnenolone, as an example of more polar steroids, elutes much later. In a systematic study of the chromatography of steroids on Sephadex LH-20, the separation of cholesterol from more polar steroids with this same solvent system has been shown previously[11].

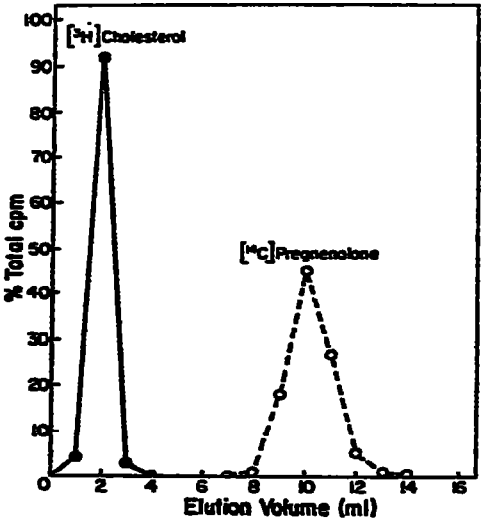

Fig. 1. The elution profile of [^{3}H]cholesterol and [^{14}C]pregnenolone on Sephadex LH-20 under conditions for purification of radioactive cholesterol. The ordinate represents the percentage of total ^{3}H (●) or ^{14}C (○) radioactivity applied onto the column. Elution is with isooctane–benzene–methanol (90:5:5). See Materials and methods for other details of the chromatography procedure.

The procedure described above offers several advantages for cholesterol purification over those currently used: *e.g.*, TLC, Celite column chromatography. The method is simple and requires a minimal amount of gel. Cholesterol is rapidly eluted with quantitative recovery while separation from more polar steroids and presumably from radiolysis products is maintained. After purification of [^{3}H]-

cholesterol in this system, > 95% of the ^3H migrates together with carrier cholesterol in the TLC systems mentioned in Materials and methods.

Pregnenolone separation from cholesterol

In the Sephadex LH-20 system described above, pregnenolone is eluted with 50% methanol while cholesterol remains adsorbed on the column (Fig. 2). We have settled on a concentration of 50% methanol after testing lower and higher proportions. Decreasing the proportion of methanol delays the elution of pregnenolone while increasing this proportion results in the elution of cholesterol close to or together with pregnenolone. The elution profiles of pregnenolone extracted from incubation media with or without detergent Tween 20 are not significantly different.

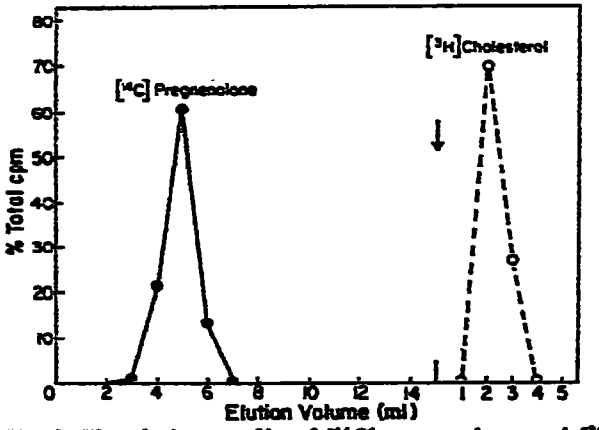

Fig. 2. The elution profile of [^{14}C]pregnenolone and [^3H]cholesterol on Sephadex LH-20 used for assay of cholesterol side chain cleavage. The ordinate represents the percentage of total ^3H (○) or ^{14}C (●) radioactivity eluted from the column. The first elution solvent was 50% methanol, the arrow marks the beginning of 100% methanol elution. See Materials and methods for other details of the chromatography procedure.

The distribution of [^{14}C]pregnenolone and [^3H]cholesterol counts between the elution fractions as collected under standard assay conditions is shown in Table I. The key point to note is that fraction II discriminates highly between cholesterol and pregnenolone so that 0.5% conversion of product can be accurately measured. This value is far below the approximately 10% conversion that occurs in 4 min during the linear portion of the reaction time course (Fig. 3). If the substrate, [^3H]cholesterol, is not highly pure, the low blank value (0.08%) may increase and consequently lower the sensitivity of the assay.

Since the recovery of pregnenolone is not quantitative (Table I), the use of [^{14}C]pregnenolone as a recovery standard is obligatory. Although the recovery of the steroids in the methylene chloride extracts are > 95%, the dissolution of the dried extract in 40% methanol for application onto the column reduces the recoveries. However, these recoveries can be accurately quantitated so that this does not pose a problem. Higher concentrations of methanol or the presence of small amounts of other organic solvents, *e.g.*, methylene chloride or benzene in the sample applied to the column interfere with the separation.

TABLE I

SEPARATION OF RADIOLABELED PREGNENOLONE AND CHOLESTEROL BY SEPHADEX LH-20 MINICOLUMN CHROMATOGRAPHY

The standard assay incubation solutions excepting cytochrome $P\text{-}450_{scc}$ were extracted, and chromatographed as described in Materials and methods. Each solution contained $2.16 \cdot 10^5$ cpm, 40 nmol [^{3}H]cholesterol and 1600 cpm [^{14}C]pregnenolone. The values are the mean ±S.D. of six determinations.

Elution fraction	*[^{14}C]Pregnenolone (% total cpm)*	*[^{3}H]Cholesterol (% total cpm)*
(I) First 50% methanol elution (3 ml)	1.00 ± 0.10	0.70 ± 0.04
(II) Second 50% methanol elution (3 ml)	84.00 ± 6.10	0.08 ± 0.01
(III) 100% methanol elution (5 ml)	4.40 ± 1.80	70.00 ± 5.20

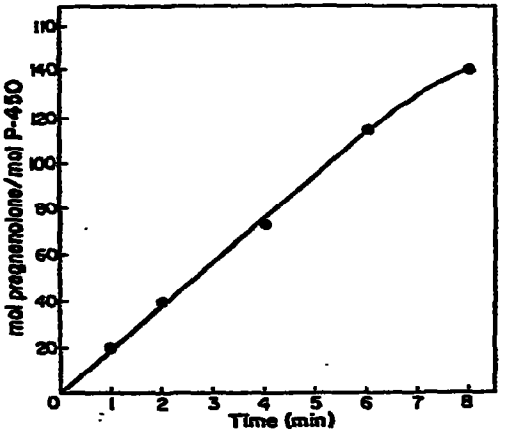

Fig. 3. [^{3}H]cholesterol conversion to [^{3}H]pregnenolone as a function of time under standard assay conditions. The reactions were carried out in 27 m*M* K phosphate buffer, pH 7.2, 10 m*M* KCl, 0.3% Tween 20 with 200 μM cholesterol, 270 n*M* $P\text{-}450_{scc}$, 300 n*M* adrenodoxin reductase, 4.5 μM adrenodoxin and 1 m*M* NADPH in a final volume of 0.2 ml at 37°. The final concentrations take into account the buffer and salt concentrations present in the enzyme preparations. The separation of [^{3}H]pregnenolone from [^{3}H]cholesterol and its quantitation is described in Materials and methods.

The Sephadex LH-20 procedure described here can serve as an alternative to TLC. It has considerable advantages over TLC; *e.g.*, the application of sample is faster, the product to be quantitated elutes for counting and does not require manual handling of radioactive chromatography media as in TLC scraping. Furthermore, this system does not have the disadvantages common to some Sephadex LH-20 chromatography systems. The column is short, the packing quick, and the solvent not as expensive and noxious as hydrocarbon solvents that are commonly used. Most significantly, the method gives reproducible data so that the average difference of the replicates from their mean is 3.5% in the same experiment.

The standard assay conditions are based on saturating concentrations of adrenodoxin, adrenodoxin reductase and optimal ionic strength[12]. The presence of Tween 20 in the incubation solution, in addition to increasing the solubility of the substrate cholesterol, protects cytochrome $P\text{-}450_{scc}$ from denaturation[6]. NADPH

can be used as electron donor without a generating system because, as indicated in Fig. 3, the reaction rate is linear for at least 5 min. The mean rate under standard assay conditions is 17.6 nmol pregnenolone produced per min per nmol cytochrome $P\text{-}450_{scc}$ with an interassay coefficient of variation (for incubations with different preparations on different days) of 4.7%.

The sensitivity, reproducibility and ease of this assay suggest that it may be the method of choice for routine assessment of activity of purified cytochrome $P\text{-}450_{scc}$ preparations. In addition, it may prove useful for kinetic studies with mitochondria when separation and quantitation of radioactive pregnenolone is required. The same separation procedure may also be useful for radioimmunoassay of pregnenolone where a separation from interfering substances is necessary.

ACKNOWLEDGEMENT

This work was supported by NIH Grant AM18585.

REFERENCES

1 E. R. Simpson, *Mol. Cell. Endocrinol.*, 13 (1979) 213–227.
2 D. G. Young and P. F. Hall, *Biochemistry*, 8 (1969) 2987–2997.
3 L. Bjorkhem and K.-E. Karlmar, *Anal. Biochem.*, 68 (1975) 404–414.
4 B. A. Teicher, N. Koizumi, M. Koreeda, M. Shikita and P. Talalay, *Eur. J. Biochem.*, 91 (1978) 11–19.
5 E. R. Simpson and G. S. Boyd, *Eur. J. Biochem.*, 2 (1967) 275–285.
6 O. Takikawa, T. Gomi, K. Suhara, E. Itagaki, S. Takemori and M. Katagiri, *Arch. Biochem. Biophys.*, 190 (1978) 300–306.
7 R. B. Hochberg, T. A. vander Hoeven, M. Welch and S. Lieberman, *Biochemistry*, 13 (1974) 603–609.
8 M. E. Haberland and J. A. Reynolds, *Proc. Nat. Acad. Sci. U.S.*, 70 (1973) 2313–2316.
9 W. H. Orme-Johnson and H. Beinert, *J. Biol. Chem.*, 244 (1969) 6143–6148.
10 A. Hiwatashi, Y. Ichikawa, N. Maruya, T. Yamano and K. Aki, *Biochemistry*, 15 (1976) 3082–3090.
11 B. R. Carr, G. Mikhail and G. L. Flickinger, *Endocrinology*, 33 (1971) 358–360.
12 I. Hanukoglu and C. R. Jefcoate, *J. Biol. Chem.*, 225 (1980) in press.