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A Simple and Precise Assay of the Enzymatic Conversion of Cholesterol into Pregnenolone†

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ABSTRACT: A simple and precise assay is described for measurement of the enzymatic conversion of cholesterol into the C_{21} steroid, pregnenolone. This procedure determines quantitatively the amount of the isotopically labeled C_6 fragment (isocaproic acid) formed from cholesterol, when the sterol substrate bears a radioisotope, ^{14}C or ^{3}H, in the side chain. The method is based upon the fact that the substrate is separated from the C_6 product by percolation through a column of alumina. The unreacted sterol is adsorbed out of the aqueous incubation medium by the adsorbant, whereas the small molecular weight fragment filters through. The radioactivity in the eluate serves as a direct measure of the cleavage enzyme activity and thereby provides the basis for a rapid assay which is convenient for the study of many aspects of this important enzyme.

The biosynthetic processes, by which steroid hormones are made from cholesterol, all begin with cleavage of a C_6 fragment from the side chain of the sterol. Most of the other enzymes involved in steroid hormone formation, *e.g.*, hydroxylases and Δ^5-3β-hydroxysteroid dehydrogenase–isomerase etc., proceed at rates far faster than that characterizing the side-chain cleavage of cholesterol (Koritz and Kumar, 1970). It is natural, therefore, that this rate-limiting scission is generally considered to be the principal step at which the control of the rate of hormone synthesis is exerted. The locus of action of the various trophic hormones (ACTH, LH,[1] etc.) is usually thought to be at the reaction, cholesterol → pregnenolone + isocaproic acid (Garren, 1968).

Consequently, it is evident that a rapid, easy, and sensitive assay is of great value for a study of the detailed nature of the reaction and of the factors which influence it. Although many assays have been described, none combines adequate sensitivity with ease of manipulation. In those assays which use nonradioactive cholesterol (either of endogenous or exogenous origin) as a substrate, the pregnenolone[1] formed was measured colorimetrically (Koritz, 1962), by gas–liquid chromatography (Young and Hall, 1969), by radioimmunoassay (Bermudez *et al.*, 1970), or by the appearance of DPNH[1] that occurs when pregnenolone (isolated by chromatography) is oxidized enzymatically to progesterone (Simpson *et al.*, 1972). In those determinations in which radioactive cholesterol was used as a substrate, measurement of the isotope associated with more polar products (Halkerston *et al.*, 1961), with specific C_{21} steroids after chromatography (Simpson and Boyd, 1967), or with the labeled C_6 side-chain fragment [determined by gas–liquid chromatography (Burstein *et al.*, 1971), by extraction (Raggatt and Whitehouse, 1966), by stream distillation (Constantopoulos and Tchen, 1961), or by difference following the removal of the volatile fragment by distillation (Kimura *et al.*, 1966; Doering and Clayton, 1969)] served as the end point in the analysis. Most of these methods are insensitive, and almost all are time consuming.

The method presented in this paper measures directly the radioactive C_6 fragment formed from the cleavage of [26-^{14}C]cholesterol, by a procedure that is both rapid and simple. After the enzyme reaction is arrested by dilution with an alkaline buffer containing $HgCl_2$, the entire mixture is poured onto a small column of aluminum oxide. The small molecular weight product percolates through with the buffer while the unchanged radioactive cholesterol is adsorbed out of the solution, and is retained on the column. The ^{14}C in the eluate was found to be associated only with isocaproic acid, which is produced in stoichiometric quantities with pregnenolone when cholesterol is used as a substrate or with pregnenolone sulfate when cholesterol sulfate is the precursor. The ^{14}C in the eluate is thus a quantitative measure of the extent of the reaction and may be used as the basis of an assay. The method is simple and so may readily monitor kinetic experiments in which consecutive samples can be taken only seconds apart.

Experimental Procedure

Materials and Methods

Labeled Substrates. [26-^{14}C]Cholesterol (45 Ci/mol) and [1,2-^{3}H]cholesterol (45 Ci/mmol) (purchased from New En-

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[1] Abbreviations used are: ACTH, adrenocorticotrophic hormone; LH, luteinizing hormone; Et_3N, triethylammonium; pregnenolone, 3β-hydroxy-5-pregnen-20-one; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

TABLE I: Chromatography Systems.[a]

System	Description
C-1[b]	Isooctane–toluene–methanol–1-propanol–water (3:1:4:1:0.5)
C-2[b]	Isooctane–toluene–methanol–1-propanol–water (2:2:4:1:1.5)
C-3	Isooctane–benzene–2-butanone–methanol–0.2 M triethylammonium sulfate (7:2:1:3.5:3)
C-4	Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1 M $NaHCO_3$ (4.5:1:2:2:3)
C-5	Isooctane–methanol–water (10:9:1)
C-6	Isooctane–benzene–acetone–0.2 M triethylammonium sulfate (1:1:1:2)
C-7	Isooctane–ethyl acetate–*tert*-butyl alcohol–methanol–1 M NH_4OH (2:4:2:2:3)
P-1	1-Butanol–1.5 M NH_4OH (1:1)
T-1	Benzene–ethanol–6 M NH_4OH (13.5:7.5:4)

[a] C = column–Celite partition system; P = paper; T = thin layer–silica gel GF. [b] Reverse phase.

gland Nuclear Corp.) were acetylated, and each acetate was purified by chromatography on Celite in system C-1 (Table I). The products were saponified and chromatographed on Celite in system C-2, after which they were ready for use as substrates. [24,25-^{3}H]Cholesterol (11 Ci/mmol) was prepared by the reduction of desmosterol by tritium gas with triphenylphosphinechlororhodium in dioxane (Burstein *et al.*, 1971). After removal of the catalyst, the solution was taken to dryness and the residue was redissolved in ethanol. The solvent was evaporated under a stream of N_2 and the process was repeated until the labile tritium was completely removed. The residue was chromatographed on alumina (6% H_2O) from which cholesterol was eluted with hexane–benzene (1:1). Finally, the sample was chromatographed on Celite in system C-2. Radioactive cholesterol sufate was synthesized from these compounds with pyridine–sulfur trioxide, by the procedure previously described (Roberts *et al.*, 1964). It was converted into its Et_3N^+ salt, purified by chromatography on Celite in system C-3 (Mickan *et al.*, 1969), and was then converted into its sodium salt and rechromatographed on system C-4. [7-^{3}H]Pregnenolone (12 Ci/mmol) was purchased from New England Nuclear Corp. and purified by chromatography in system C-5. [7-^{3}H]Pregnenolone sulfate was prepared in the usual manner (Calvin *et al.*, 1963) and chromatographed as its Et_3N salt in system C-6 and as its sodium salt in system C-7.

[1-^{14}C]Isocaproic acid (1 Ci/mol) (Mallinckrodt Nuclear) was purified by thin layer chromatography in system T-1 and by paper chromatography in system P-1. [^{3}H]Isocaproic acid (19 Ci/mol) was prepared and purified by the method of Hembree *et al.* (1973) which involved exposure of the sodium salt of the acid to tritium gas activated by a microwave discharge. Before use, the sample was rechromatographed on paper in system P-1. [^{3}H]Water (18 mCi/mol) was purchased from New England Nuclear Corp. and diluted to approximately 5–10 μCi/ml with H_2O. This sample was distilled before use.

Radiochemical homogeneity of a tracer was determined, where applicable, by diluting an aliquot of the tracer with an accurately weighed quantity of carrier. The mixture was then recrystallized. Only when the specific activity of the recrystallized product was equal, within experimental error, to that of the original mixture was the tracer considered to be devoid of contaminants and acceptable for further use. Steroid sulfates were crystallized as their Et_3N salts. The isocaproic acid tracer, after combination with carrier, was converted into its anilide and this derivative was chromatographed on silica gel, as previously described (Hochberg *et al.*, 1971), and recrystallized from acetone–petroleum ether.

Solubilization of Sterol Substrates. When cholesterol or its sulfate was to be used as substrate, the sterol was dissolved in acetone containing Tween 80 (Sigma Corp.). The volatile solvent was evaporated under a stream of N_2. The concentration of each sterol was so chosen that 0.1 ml of the following solution contained 20 μg of Tween 80 and usually 6 nmol (~400,000 cpm) of the sterol substrate. The Tween solution was diluted with an appropriate amount of 0.1 M Tris buffer (pH 7.4). To ensure dispersal, the mixture was sonicated three times for 20 sec at room temperature with a micro tip (Ultrasonics Inc., Model W1850) at 60 W. Aliquots of the mixture were counted for radioactivity and in this way the amount of solubilized sterol was determined. This was usually found to be greater than 90% of the added substrate. This sonicated solution was further diluted with Tris buffer to the final concentration of 3 nmol of 26-^{14}C-labeled steroid (~200,000 cpm) and 10 μg of Tween 80 per 0.1 ml. In most cases 0.1 ml of the final solution was used in the assay. These solutions were kept at room temperature and were used within 3–4 hr of preparation. However, they remain dispersed for at least 24 hr. The amount of Tween 80 is critical since an excess inhibits the reaction and increases the blank in the assay. When the concentration of Tween 80 is increased from 10 to 30 μg/ml of incubation mixture there is no significant change in the velocity of the cleavage reaction. When the influence of other nonpolar compounds on the rate of cleavage was tested, these substances were solubilized in the same manner. The final concentration of Tween 80 in incubations containing such compounds together with cholesterol (or its sulfate) was 20 μg/ml.

Preparation of an Acetone Powder from Bovine Adrenal Mitochondria. Tissue was scraped from the capsules of fresh, defatted, demedullated, bovine adrenocortical glands, following which it was homogenized in a Potter-Elvejhem glass homogenizer in a medium (20% weight of tissue/volume of medium) which was 0.25 M in sucrose, 0.01 M in Tris (pH 7.4), and 0.001 M in EDTA. If necessary, the pH was adjusted to 7.4 with 1 N NaOH. The homogenate was centrifuged at 650*g* for 10 min and the pellet obtained was resuspended in the medium (10% of the original volume) and spun again at 650*g* for 10 min. The resulting supernatant was combined with the first supernatant and the mixture was centrifuged at 8000*g* for 15 min. The new pellet was washed by resuspending it in 0.2 vol of the isolation medium and centrifuging at 8000*g* for 15 min. The washing process was repeated a second time. Then the pellet was resuspended in the medium and spun for 15 min at 650*g*. The supernatant so obtained was centrifuged at 10,000*g* for 15 min, and the resulting mitochondrial pellet was suspended in water. Twenty volumes of acetone (−20°) were added and the precipitate which formed was filtered and washed with acetone (−20°) and finally with ether (−20°). The powder was dried *in vacuo* for 30 min and then stored at −20°.

Assay Procedure. The acetone powder was suspended in 0.1 M Tris (pH 7.4), at a concentration of ~2 mg of protein/ml, and the mixture was sonicated, at 60 W using a microtip, three times at 0°, for 30 sec each time. The resulting suspen-

TABLE II: Adsorption Characteristics of Alumina Columns.

Substance	cpm Added to Alumina Column	cpm in Filtrate after Percolation	% cpm in Filtrate
[26-^{14}C]Cholesterol	25,170 (9)[a]	35 ± 7	0.14
[26-^{14}C]Cholesterol sulfate	27,630 (10)	11 ± 4	0.04
[1-^{14}C]Isocaproic acid	21,909 ± 375[b] (5)	21,795 ± 363	99.5
[^{3}H]H_2O	49,534 ± 791 (5)	49,766 ± 1188	100.5

[a] Number of samples. [b] Standard deviation.

sion was clarified by centrifugation at 8000*g* for 15 min. The supernatant was analyzed for cytochrome P-450 (Omura and Sato, 1964) and for protein (Lowry *et al.*, 1951), the content of which was about one-third to one-quarter of the weight of the powder. The concentration of cytochrome P-450 varied between 0.2 and 0.6 nm/mg of protein. This solution was then diluted with 0.1 M Tris so that the final concentration of protein was 200–400 μg/0.8 ml. The enzyme solution was kept in an ice bath until just prior to the incubation and was usually used within 2–3 hr after preparation.

TPNH,[1] a necessary cofactor for the side-chain cleavage enzyme complex, was supplied in the form of a generating system consisting of: TPN, 0.75 mg; glucose 6-phosphate, 2.5 mg; glucose-6-phosphate dehydrogenase, 0.5 U; $MgCl_2$, 95 μg (all dissolved in 0.1 ml of phosphate buffer, 0.1 M, pH 7.4). The generating system is kept in an ice bath and warmed to room temperature approximately 5 min before use.

The complete incubation mixture consisted of 0.1 ml of the sterol suspension (10 μg of Tween 80), 0.8 ml of the acetone powder suspension, 0.1 ml of the TPNH generating system, and an internal standard consisting of 100,000 cpm of either [^{3}H]water or [^{3}H]isocaproic acid. The final incubation volume of 1.0 ml was contained in a 10 × 75 mm test tube. The sterol and enzyme solutions were first incubated together at 30° for 2 min and the reaction was started by the addition of 0.1 ml of the generating system kept at 30°. The sample was immediately mixed and a 0.2-ml aliquot was quickly withdrawn. It, like all of the subsequent samples, was added to 1.5 ml of an ice-cold solution of $HgCl_2$ (0.001 M) in a glycine (0.05 M)–NaOH buffer (pH 9.5) to stop the reaction. Subsequent samples, incubated at 30° in a shaking water bath, were withdrawn at desired intervals, usually 2 min. All samples were removed with an automatic pipet (LabCages Inc.), but accurate pipetting was not necessary because of the internal standard. When the time points were 2 min apart, eight samples were easily run concurrently. In this instance, the samples were 10 sec out of phase. If longer time intervals were used, many more samples could be run simultaneously.

The diluted aliquots were then filtered through a microcolumn of alumina, a process that almost completely retains the sterols while permitting the side-chain fragment to pass through unabsorbed. Approximately 500 ± 100 mg of alumina was scopped into a glass column which was made from a disposable Pasteur pipet (145 mm × 5 mm i.d.) plugged with glass wool. The absorbent was a 1:1 mixture of two brands of alumina [thin layer grade (Merck) (mesh < 40 μ) and column grade (Woelm) (mesh > 60 μ)]. The diluted aliquot, when poured into this mixture, percolated through the column slowly. The filtration was complete by the next morning. If the filtration is too rapid, small, varying amounts of sterol pass through the column. Thin-layer grade alumina could also be used, but, with it, the filtration was occasionally not completed by morning. Aliquots (1 ml) of the filtrate were counted in 10 ml of a dioxane-based phosphor solution, prepared as previously described (Hochberg *et al.*, 1971), and the ^{14}C content of the sample was a measure of the amount of isocaproic acid formed. Samples were counted in a Packard Tri-Carb Scintillator, Model 3375. When ^{3}H and ^{14}C were counted together, the efficiency for ^{14}C was 59%, and the overlap into the ^{3}H channel was 12%. The efficiency for ^{3}H was 38% and the overlap into the ^{14}C channel was 0.03%. These counts were corrected by the method described by Okita *et al.* (1957).

Aliquots were taken from the incubation mixture periodically. By so doing, an internal control to check both the velocity and the linearity of the reaction was obtained, and the zero time value verified. The velocity was determined by linear regression analysis (using a Hewlett-Packard, desk-top calculator) which provided values that agreed well with those obtained by visual examination of graphed data.

Results

Validation of Assay Procedure. FILTRATION THROUGH ALUMINA. That filtration through alumina was a suitable means of separating the sterol substrates from their side-chain fragments was demonstrated in the following way. Several samples each of [^{14}C]isocaproic acid, [^{14}C]cholesterol sulfate, [^{14}C]cholesterol, and [^{3}H]water were diluted with the appropriate components to simulate an incubation mixture. A 0.2-ml portion of each solution was diluted with 1.5 ml of mercuric chloride–glycine buffer diluent and the mixture was percolated through the alumina column as described above. Aliquots were taken both before and after filtration, and the radioactivities of these samples determined. The data in Table II show that virtually all of the sterol was adsorbed onto the column, whereas amounts of labeled isocaproic acid and [^{3}H]water in the eluate were identical with those in the simulated incubation mixtures. The values shown in Table II are from a representative experiment and, while the results are typical, experiments in which no sterol at all is eluted from the columns were frequently encountered.

IDENTIFICATION OF LABELED ISOCAPROIC ACID IN THE FILTRATE FROM ALUMINA COLUMNS. [26-^{14}C]Cholesterol (1.25 × 10^6 cpm) was incubated in 5 ml of incubation mixture, with the same amounts of cofactor, detergent, and enzyme preparation described above. Samples (0.2 ml) were taken every 2 min for 8 min. These aliquots were assayed and a normal linear increase in radioactivity was found. In addition, at 6 min, 4 ml of the mixture was withdrawn and treated with 26 ml of the glycine buffer–$HgCl_2$ solution. Several 1.7-ml samples of the resultant solution were each filtered through individual microalumina columns. The eluates were combined and 200 μl (184.5 mg) of unlabeled isocaproic acid was added as

TABLE III: Crystallization Data.[a]

Sample	Fraction	Weight (mg)	cpm	Sp Act. (cpm/mg)
Isocaproic acid anilide	X-1	0.976	148	152
	X-2	1.280	198	155
	X-3	1.359	216	159
	ML-3	0.554	84	152
Pregnenolone	X-1	1.325	5,397	4,072
	ML-1	0.959	3,634	3,791
	X-2	1.544	6,371	4,127
	ML-2	1.684	6,931	4,117
Pregnenolone sulfate	X-1	0.673	9,168	13,623
	ML-1	0.539	7,082	13,139
	X-2	0.967	13,193	13,643
	X-3	0.803	10,605	13,207

[a] X-*n*, crystalline product from the *n*th crystallization. ML-*n*, residue left in the mother liquor from the *n*th crystallization.

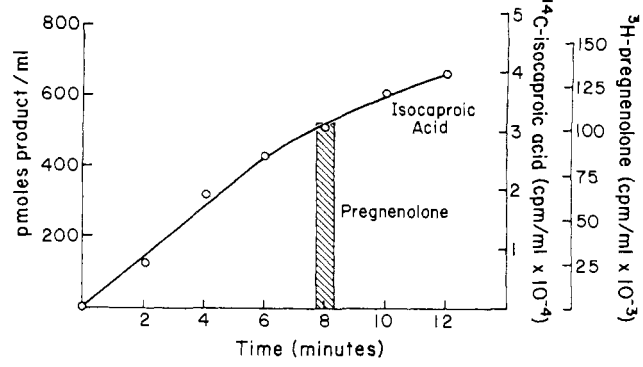

FIGURE 1: Stoichiometry of enzymatically produced [^{3}H]pregnenolone and [^{14}C]isocaproic acid. ^{3}H/^{14}C ratio of the substrate [26-^{14}C, 1,2-^{3}H]cholesterol = 3.32. [^{14}C]Isocaproic acid was determined without the aid of an internal standard.

carrier. One milliliter of the combined solutions was counted and the amount of isocaproic acid present was calculated by assuming that all the ^{14}C (4150 cpm/ml) was associated with isocaproic acid. A 10-ml portion of the combined solutions was acidified and the anilide was prepared and chromatographed on silica gel as previously described (Hochberg *et al.*, 1971). The radioactive product was recrystallized three times from acetone–petroleum ether. The specific activities of the products and residues in the mother liquors are shown In Table III. From the results (average sp act. of isocaproic acid anilide, 155 cpm/mg), it would appear that all the radioactivity present in the alumina eluate was the expected C_6 acid. The total counts per minute of [^{14}C]isocaproic acid = ($a \times b \times R$), where a = specific activity of the purified anilide, b = weight in milligrams of added unlabeled isocaproic acid, and R = ratio of the molecular weights of the anilide (191.3) and isocaproic acid (116.2). The amount of radioactivity in the [^{14}C]isocaproic acid determined in this way was 47,055 cpm; the quantity expected was 41,500 cpm.

STOICHIOMETRY. The following experiment was designed to show that the amount of the radioactivity in the column filtrate (as [^{14}C]isocaproic acid) was equivalent to the amount of pregnenolone produced in the enzymatic reaction. With two exceptions, incubation was performed as previously described. In this instance, nuclear-labeled [1,2-^{3}H]cholesterol (1,087,000 cpm) and side-chain labeled [26-^{14}C]cholesterol (327,600 cpm) (^{3}H/^{14}C = 3.32) were incubated in 2.5 ml of the incubation mixture. In addition, 10 μg of 17β-hydroxy-4,4,17-trimethyl-3-oxoandrost-5-ene-2α-carbonitrile (cyano ketone) (Goldman *et al.*, 1965) was added to the incubation to inhibit the conversion of pregnenolone into progesterone. Samples (0.2 ml) of the incubation mixture were removed every 2 min for 12 min and each sample was analyzed for filterable ^{14}C side-chain fragments. After 8 min of incubation, an aliquot of 1 ml was added to 10 ml of methanol containing 25 mg of pregnenolone.

The radioactivity in the side-chain cleavage fragments produced during the incubation was determined and is shown in Figure 1. The counts per minute of [^{14}C]isocaproic acid present in 0.2 ml at 8 min of incubation was 6123. From the original ^{3}H/^{14}C ratio (3.32) of the substrates used, 1 ml of the reaction mixture taken after 8 min of incubation should theoretically contain 101,642 cpm of [^{3}H]pregnenolone.

The 1-ml incubation sample, added to the methanolic solution of 25 mg of unlabeled pregnenolone, was clarified by filtration and the filtrate evaporated to dryness. The residue was chromatographed on a 50-g Celite column in system C-5. The column fractions in the region where progesterone would appear were devoid of significant amounts of radioactivity. Those fractions containing the carrier pregnenolone were combined, evaporated, and recrystallized to constant specific activity from acetone–petroleum ether (Table III). The specific activity of the final product was 4127 cpm/mg, from which it can be calculated that the total amount of tritium in the [^{3}H]pregnenolone formed was 103,175 cpm (Figure 1).

To ensure the stoichiometry of the assay when cholesterol sulfate is used as substrate, the following experiment was carried out. [1,2-^{3}H]Cholesterol sulfate, 1.55×10^{6} cpm, and [26-^{14}C]cholesterol sulfate, 6.96×10^{5} cpm (^{3}H/^{14}C ratio = 2.23), were incubated together for 8 min in 3.0 ml of incubation solution. Samples (0.2 ml) were taken every 2 min and, at 6 min, a 2-ml aliquot was diluted with 10 ml of methanol containing 9.2 mg of the Et_3N salt of pregnenolone sulfate. The alcoholic solution was clarified by filtration and then evaporated to dryness. The residue was converted into the Et_3N salt (Mickan *et al.*, 1969) and chromatographed on a 20-g Celite column using system C-6. The fractions containing the Et_3N salt of pregnenolone sulfate were crystallized from methanol to constant specific activity (13,207 cpm/mg) (Table III). From this specific activity, the total counts per minute of tritium associated with the synthesized [^{3}H]pregnenolone sulfate can be calculated (13,207 cpm/mg $\times$ 9.2 mg) to be 125,120 cpm. At 6 min, the 0.2-ml aliquot contained 5525 cpm of ^{14}C, as isocaproic acid. Based on the ^{3}H/^{14}C ratio (2.23) of the cholesterol sulfate used as substrate, the value for the [^{14}C]isocaproic acid content corresponds to 123,000 cpm of [^{3}H]pregnenolone sulfate present in the 2-ml aliquot sampled after 6 min of incubation. This result is illustrated in Figure 2.

OTHER CONTROLS. Several control experiments were carried out to authenticate the assay method further. For example, when incubations were performed in the absence of a TPNH generating system, or a viable acetone powder, no ^{14}C was detectable in the eluent from the column (Table IV). When known inhibitors of the reaction, such as aminoglutethimide (Cash *et al.*, 1967) or the end-product inhibitor, pregnenolone (Koritz and Hall, 1964), were included in the incubation

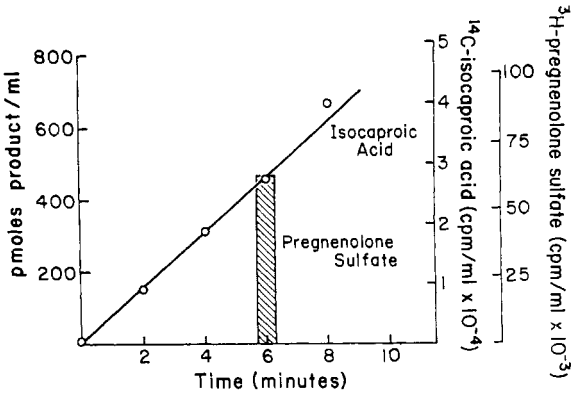

FIGURE 2: Stoichiometry of enzymatically produced [^{3}H]pregnenolone sulfate and [^{14}C]isocaproic acid. ^{3}H/^{14}C ratio of the substrate [26-^{14}C,1,2-^{3}H]cholesterol sulfate = 2.23. [^{14}C]Isocaproic acid was determined without the aid of an internal standard.

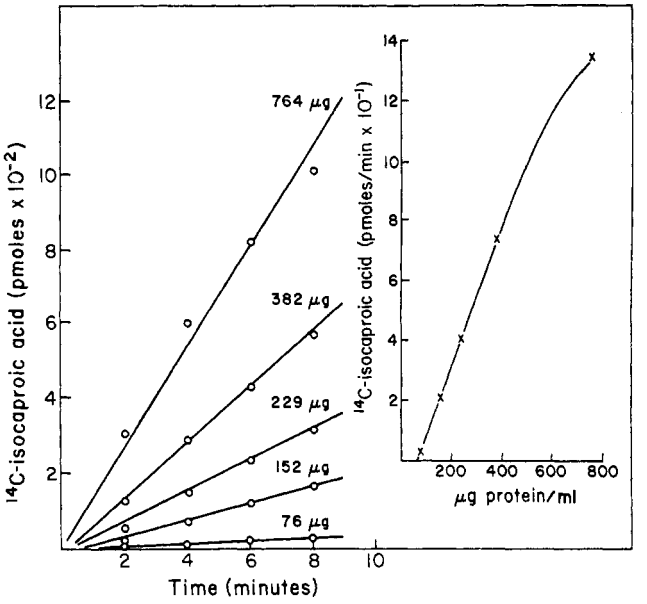

FIGURE 3: Effect of enzyme concentration on rate of isocaproic acid formation. Values given as micrograms refer to protein concentration per milliliter. The velocities used for the graph in the insert were calculated by linear regression analysis of the kinetic data shown.

mixture, less than the theoretical amount of [^{14}C]isocaproic acid appeared in the filtrate. As expected, when the concentration of the acetone powder was altered, the assay was able to detect variation in the rate of cleavage (Figure 3). The dependence of the rate of reaction on protein concentration falls below expected values at concentrations below 100 and above 800 μg/ml. Because of this decrease in rate at low concentrations of protein, the line in the inset in Figure 3 intercepts the abscissa at values greater than zero. In other experiments an even more precipitous fall in the rate than that illustrated has been observed. This decrease may be due to the coincidental dilution of another essential factor. For example, nonheme iron protein is required for the reduction of the cytochrome P-450 from adrenal mitochondria. The addition of nonheme iron protein to acetone powders of this subcellular particle has been previously reported (Burstein *et al.*, 1972), presumably in order to support side-chain cleavage enzyme activity at maximal rates. This protein, present in limiting amounts, is an example of such a factor.

That the rate of reaction observed in this study is lower than expected at high concentration of protein (greater than 800 μg/ml) is probably due to limiting substrate concentrations although another possibility is that the endogenous sterol concentration also increases with the protein concentration and this dilutes the radioactive substrate. In this event, the rate would appear to be decreased. The former explanation seems more probable since at high protein concentrations the rate of reaction observed is not linear. It decreases with time, suggesting the limited availability of substrate. It is important to note that the rate data in this article were calculated solely from the specific activities of the labeled substrates. It was assumed that if any endogenous cholesterol were in the acetone powders, it would be present in quantities much smaller than those of the radioactive substrates.

In Figure 4, the dependence of the rate of formation of isocaproic acid upon the order of the addition of the substrate is illustrated. In one experiment (B), the reaction was begun by adding cholesterol last. In the other instance (A), the incubation mixture was preincubated with [^{14}C]cholesterol for 2 min before the addition of TPNH. Samples were drawn only seconds apart and the results demonstrate that there is a lag phase of almost 2 min when cholesterol is added last. When cholesterol is allowed to equilibrate with the enzyme preparation before the addition of the cofactor, the reaction immediately commences upon addition of the latter.

TABLE IV: Effect of Alterations on the Cholesterol Side-Chain Cleavage Assay.

Additions or Deletions	Velocity (cpm/min)	% Control
Complete	2100	100
− TPNH generating system	0	0
− Acetone powder	0	0
Boiled acetone powder	0	0
Complete + 1.5 × 10^{-5} M aminoglutethimide	1240	59
Complete + 1.5 × 10^{-5} M pregnenolone	1360	65

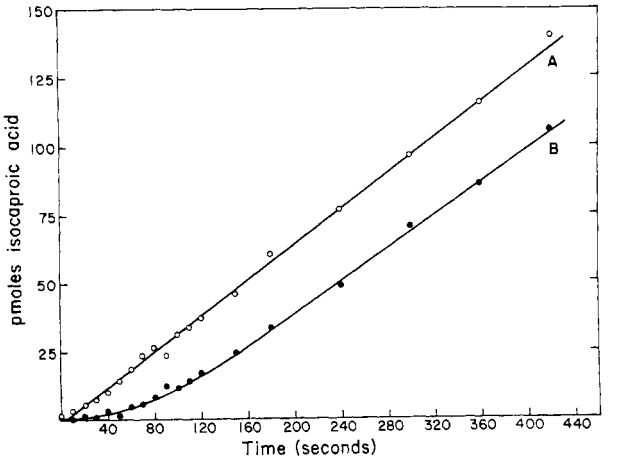

FIGURE 4: Effect of preincubation on the reaction kinetics. The data used for curve A were obtained by preincubating cholesterol with acetone powder for 2 min before starting the reaction. The data used for curve B were obtained by adding cholesterol after the TPNH and the enzyme preparation had been mixed.

PRECISION. In order to assess precision, the assay was performed at three different enzyme concentrations, each determination in quadruplicate. The average coefficient of variation of the velocity estimated in this way was 4%.

Discussion

A precise assay for the enzyme that cleaves the side chain of cholesterol during its conversion into pregnenolone is described in this report. This assay is based on the fact that alumina columns will adsorb quantitatively cholesterol dissolved or suspended in an aqueous medium whereas the smaller molecular weight fragment, isocaproic acid, is not retained by the column. If the atoms of the side chain (C_{22}–C_{26}) of the substrate, cholesterol, are labeled with either ^3H or ^{14}C, the radioactive isocaproic acid formed serves as a measure of the reaction. Nuclear labeled cholesterol cannot be used for this assay, for the labeled pregnenolone formed is not quantitatively adsorbed by the alumina column. However, side-chain labeled cholesterol sulfate, the only known ester of cholesterol capable of enzymatic conversion into a C_{21} steroid (Roberts *et al.*, 1967), can also be assayed by this technique. Indeed, the sterol sulfate has some advantages as a substrate since a lower blank, and usually a higher rate of conversion, has been obtained with it.

The data presented in this report were obtained, for the most part, from measurements made on a zero time sample and four others taken at 2-min intervals. Ordinarily, determinations made at only two time points (3 and 6 min) may suffice for an accurate analysis but in such cases it is necessary to standardize the procedure carefully.

When the reaction rate is very fast, the value taken at zero time may not coincide with the origin. A straight line, drawn through the remaining points, may still give an accurate estimate of the rate. Such rates are reproducible in replicate determinations and are consistent with values determined at lower enzyme concentrations under which conditions the rate curves pass through the origin. The assay is performed at 30° since the reaction seems to follow straight line kinetics better at this temperature than at 37°, as has already been reported (Doering and Clayton, 1969). This is probably due to the instability of the enzyme at higher temperatures. In general, incubations were carried out for not longer than 8 min because beyond that the rate decreased. In one experiment, the enzyme was preincubated without cholesterol for varying times at 30°, and then assayed in the usual way. After 5 min of preincubation, 96% of the enzyme activity remained; after 10 min of preincubation, 90% remained; 20 min, 83%; 30 min, 75%; 60 min, 66%; and 90 min, 55%. This instability of the enzyme may be an important consideration when assaying relatively inactive preparations of the enzyme which require longer periods of incubation.

The assay as described has a small blank and has great sensitivity which obviously is dependent principally upon the specific activity of the substrate. As shown in Figure 3, the amount of pregnenolone produced from cholesterol when the sterol was incubated with 76 μg of protein for 2 min was 3.8 pmol which corresponds to a yield of only 0.12%. Nevertheless, this value was easily estimated and differentiated from that of the zero time sample which was completely devoid of ^{14}C.

The use of [^3H]H_2O or [^3H]isocaproic acid as internal standards to correct for recovery allows for ease of sampling without, in any way, altering the velocity of the reaction. When [24,25-^3H]cholesterol is used, [^{14}C]isocaproic acid, which is commercially available, can be used as an internal standard. In fact, any isotopically labeled compound that is completely excluded by the alumina column, and that does not interfere with the determination of tritium, can be used. Although they have not been tried, $^{35}SO_4^{2-}$, $^{32}PO_4^{2-}$, or [^{14}C]acetate are possible examples. It may be feasible to assay [26-^{14}C]cholesterol and [24,25-^3H]cholesterol sulfate side-chain cleavage in the same tube using $^{32}PO_4^{2-}$ as an internal standard.

On three occasions we have determined that all the radioactivity in the column eluate is present as isocaproic acid. This is in contrast to the findings of Burstein *et al.* (1971) who observed that both isohexyl alcohol and isocaproaldehyde are also formed, sometimes in sizable quantities, in incubations of this kind. Why this discrepancy exists is not clear but as far as the assay reported in this paper is concerned, the formation of these nonacidic C_6 fragments seems to be of little concern. Either they are oxidized to the acid under the conditions employed in the assay or they too are excluded from the alumina column. In either case, the results from the assay are unaffected since, as has been shown above, the ^{14}C content of the eluate corresponds to the amount of pregnenolone formed.

In order to obtain straight line kinetics, it is important to ensure equilibration of the enzyme and the substrate, cholesterol. As can be seen in Figure 4, preincubation of about 2 min is necessary to achieve this state. This finding differs from that of Burstein *et al.* (1972) who have reported that prior incubation of the substrate and enzyme leads to an initial burst of activity when TPNH is finally added. They found that the addition of the substrate last to the mixture of the enzyme preparation and the TPNH-generating system results in a linear reaction with only a minor lag in the rate. These investigators (Burstein *et al.*, 1972) used a substrate of high specific activity and little weight and such samples probably equilibrate rapidly with the enzyme.

The use of this assay is not limited to acetone powders of adrenal mitochondria. It has also been used on intact adrenal mitochondria (R. Hochberg *et al.*, manuscript in preparation), intact ovarian and placental mitochondria (P. Graves, V. Uzgiris, and H. A. Salhanick, personal communication), and ovarian granulosa cells grown in tissue culture (W. Miller and R. Hochberg, unpublished observations). In general, this type of assay could probably be adapted to other enzymatic processes where small hydrophilic molecules or even water are cleaved from a larger hydrophobic substrate. A few examples that might be offered are: the formation of bile acids from [26-^{14}C]- or [25-^3H]cholesterol where a C_2 fragment is formed or the hydroxylation of steroids labeled with tritium at specific carbon atoms, *e.g.*, C_1, C_{11}, C_{21}, or C_{25} when [^3H]-H_2O is formed as occurs during the biosynthesis of estrogens, corticosteroids, or vitamin D metabolites.

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Studies on the Conformation and Interactions of Elastin. Proton Magnetic Resonance of the Repeating Pentapeptide†

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ABSTRACT: Proton magnetic resonance studies have been carried out on the penta-, deca-, and pentadecapeptides of the basic sequence: L-Val-L-Pro-Gly-L-Val-Gly. Two methods—temperature dependence and methanol–trifluoroethanol solvent mixture dependence of peptide proton chemical shift—have been utilized effectively to delineate peptide protons. The two methods, the relatively high field position of the valine$_{4,9,14}$ peptide protons and the $^3J_{NH-CH}$ for the valine residues, are consistent with the presence of a β turn with the sequence L-Pro-Gly at the corners.

Elastin is the protein component of connective tissue that is largely responsible for the elastic properties of ligaments and arterial walls. Furthermore, the elastic fibers of the arterial wall are major foci for the deposition of calcium salts and lipid in the pathogenic process of arteriosclerosis (Yu and Blumenthal, 1965; Kramsch and Hollander, 1973). Elastin is an insoluble, highly cross-linked protein which until very recently has only been studied spectroscopically as fragments, called α- and β-elastin (Partridge *et al.*, 1955; Partridge and Davis, 1955), which are obtained by hot oxalic acid treatment of the purified fiber.

Recently, however, Foster *et al.* (1973) have determined the sequence of a dozen peptides obtained from tropoelastin—a soluble, noncross-linked precursor of elastin which is isolated from the aortas of copper-deficient pigs. The peptides, which represent a sum of approximately 350 residues or close to one-half of the tropoelastin molecule, exhibit repeating sequences. The repeating sequences are a tetrapeptide, -Gly-Gly-L-Val-L-Pro-; a pentapeptide, -L-Pro-Gly-L-Val-Gly-L-Val-; and a hexapeptide, L-Pro-Gly-L-Val-Gly-L-Val-L-Ala. The existence of sequences with regularly positioned glycines has been anticipated (Urry, 1972).

In the present manuscript we report proton magnetic resonance studies on repeating pentapeptides synthesized in this laboratory. The basic sequence L-Val$_1$-L-Pro$_2$-Gly$_3$-L-Val$_4$-Gly$_5$ was synthesized as were the dimer and trimer of the sequence, *i.e.*, the decapeptide and pentadecapeptide. Assignments were achieved by noting the multiplet patterns and decoupling, by noting the chemical shifts of fragments derived during the synthesis and by chemically differentiating end residues. Dependence of peptide proton chemical shifts on temperature and on methanol–trifluoroethanol solvent mixtures was utilized to delineate peptide protons as an initial step in arriving at a secondary structure. The above dependencies of chemical shift taken together with the valyl coupling constants and the relative chemical shifts of the peptide pro-

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