NEW ASSAYS FOR THE ENZYMATIC CONVERSION OF CHOLESTEROL TO PREGNENOLONE

Rabe T., Rabe D., Bierwirth A.-M., and Runnebaum B.

Abteilung für gynäkologische Endokrinologie, Universitäts-Frauenklinik Heidelberg, Voßstr. 9, D-6900 Heidelberg, West Germany

Received 1-16-81

ABSTRACT

Cholesterol side chain cleavage is determined by means of separation of (26-^{14}C)-cholesterol and its radioactively labeled side chain (1-^{14}C)-isocaproic acid. Alumina minicolumn assay (AMCA): adsorption of cholesterol from an aqueous phase by aluminium oxide, while isocaproic acid can percolate through the column. In modification of a previously described technique (1), cholesterol is quantitatively eluted by ethanol. Filter assay (FA): retention of cholesterol by a membrane filter (pore size $\leq$ 0.1 µm) while isocaproic acid can pass the filter. Two-phase scintillation assay (TPSA): pH-dependent partition of isocaproic acid between an organic scintillation mixture and an aqueous phase. The TPSA can be applied for all enzymatic reactions in which the polarity of the radioactive residue which is split off depends on pH values or when the total charge of a polar molecule is changed to an apolar state by cleaving one non-radioactive group (e.g. steroid sulfates) and vice versa.

The criteria of reliability of the test systems are described. Bovine adrenal mitochondria were incubated and the side chain cleavage of (26-^{14}C)-cholesterol was studied by the new test systems and compared to the conversion rates of (4-^{14}C)-cholesterol to its metabolites as determined by thin layer chromatography. A good agreement of all tests was found.

INTRODUCTION

The first step in the steroidogenesis of hormones formed from cholesterol is the cleavage of a C_6 fragment (isocaproaldehyde or isocaproic acid) from the side chain of the sterol. It is assumed that the cleavage of the cholesterol side chain is the rate-limiting step by which the synthesis of pregnenolone and furthermore of progesterone can be controlled (2).

This paper describes two new and one modified cholesterol side chain cleavage system based on the separation of (26-C^{14})-cholesterol and its radioactive side chain, (1-^{14}C)-isocaproic acid.

MATERIALS AND METHODS

1) Abbreviations and trivial names:

Cholesterol: 5-cholesten-3ß-ol; pregnenolone: 3ß-hydroxy-5-pregnen-20-one; progesterone: 4-pregnene-3,20-dione; isocaproic acid: 4-methyl-n-valeric acid; isocaproaldehyde: 4-methylpentan-1-al; isohexanol: 4-methylpentan-1-ol; PPO: 2,5-diphenyloxazole; Tween® 80: polyoxyethylene sorbitan mono-oleate.
Alumina minicolumn assay = AMCA; filter assay = FA; two-phase scintillation assay = TPSA; thin layer chromatography = TLC.

2) Chemicals:

(26-^{14}C)-cholesterol (53 mCi/mmol) and (4-^{14}C)-cholesterol (57 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, (Buckinghamshire, England). (1-^{14}C)-isocaproic acid, sodium salt (2-10 mCi/mmol) was obtained from CEA Ire Sorin via Isotopen Dienst West (Sprendlingen, West Germany).
NADP, disodium salt (purity 98%) (grade I), glucose-6-phosphate and glucose-6-phosphate dehydrogenase (grade I) from yeast (approx. 350 IU/mg and 1 mg/ml) were obtained from Boehringer Mannheim GmbH (Mannheim, West Germany).
Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden. Celite (Serva type 545, 20-45 µm) was purchased from Serva (Heidelberg, West Germany).
All other chemicals were of analytical grade and were provided by Merck (Darmstadt, West Germany). For TLC, silica gel 60 F_{254} pre-coated plates 20 x 20 cm (layer thickness 0.25 mm) were purchased from Merck, (Darmstadt, West Germany). Solvents used for TLC and column chromatography were freshly redistilled prior to use.

3) Purification of (26-^{14}C)-cholesterol and (4-^{14}C)-cholesterol:

(26-^{14}C)-cholesterol and (4-^{14}C)-cholesterol were purified by column chromatography on Celite (3). The purity of aliquots was checked by TLC (4).

4) Mitochondrial preparation:

The isolation of mitochondria from bovine adrenal cortex was performed as described by Hochberg et al. (1). After isolation, the mitochondrial suspension was stored at -15°C; the protein concentration of this enzyme stock solution was 10 mg/ml (Lowry et al., 5). No loss of cholesterol side chain cleavage activity could be detected over a period of several months.

5) Incubation procedure:

Incubations were carried out in 20 ml glass scintillation vials (Packard, Frankfurt, West Germany) at 37°C under air in a shaking water bath (Kötter-mann, West Germany). Each incubation contained approx. 10^6 dpm (0.48 µCi) ^{14}C-labeled cholesterol and 20 µg/ml Tween 80 as solubilizer. Tris-HCl buffer, 30 mM, pH 7.4, containing 250 mM sucrose, 1 mM $CaCl_2$ and 6.25 mM $MgCl_2$ was used as incubation medium. Cholesterol was solubilized by ultrasonication for 1 min in an ultrasonication water bath (Lehfeld, Heppenheim, West Germany). As cofactors, 8.4 mg NADP (10 µmoles), 20 mg glucose-6-phosphate (65 µmoles) and 7 IU glucose-6-phosphate dehydrogenase were added to each test flask 15 min prior to the start of the mitochondrial incubation. At the end of this preincubation at 37°C the enzymatic reactions were started by addition of 1 ml of a freshly diluted mitochondrial solution (2 mg protein per test) to give a final test volume of 10 ml. After various time intervals, aliquots of 0.2 ml were withdrawn for the determination of (26-^{14}C)-cholesterol and (1-^{14}C)-isocaproic acid as described below.

6) Description of the methods

6.1 Alumina mini column assay (AMCA): The alumina mini columns were prepared as described previously (1). As chromatography system, a glycine

(0.05 M)-NaOH buffer, pH 9.5 containing 1 mM $HgCl_2$, was used. Prior to the application of 0.2 ml aliquots, each column was rinsed with 0.75 ml of ice-cold buffer solution. After application of the aliquot, 0.75 ml buffer solution was applied to the column. The eluate (approx. 1 ml) was collected in a plastic scintillation vial (Zinsser, Frankfurt) (main eluate). The elution was complete by the next morning. After addition of 10 ml scintillation fluid, each vial was acidified with 0.1 ml 1 N HCl. Scintillation counting was performed as described below. Each column was rinsed again twice with 0.75 ml glycine-NaOH buffer. The collected eluates (1.5 ml) were counted in a similar manner (posteluates). Cholesterol adsorbed on aluminium oxide was eluted from the columns using 3 x 1.5 ml ethanol and collecting the eluate in the same scintillation vial. After reduction of the ethanol volume to 1-2 ml using a stream of nitrogen, radioactivity was counted after addition of 10 ml scintillation fluid. The impulse rates (cpm) of the main and posteluates were added. The total activity corresponds to the quantity of C_6 fragment which can percolate through the column. In some tests using serum proteins or solubilizers, one part of the cholesterol can permeate the column. This part of cholesterol in the buffer eluate can be determined by a TPSA (see 6.3): after alkalization of the previously acidified vials with 0.1 ml 2 N NaOH, the cholesterol present in the eluate can be detected on the basis of the impulse rate in the alkaline medium because the scintillation counting of cholesterol is independent of the pH value. The amount of cholesterol detected in the buffer eluate is subtracted from the isocaproic acid fraction and added to the cholesterol fraction (Fig. 3).

6.2 Filter assay (FA): The FA was performed using a multifiltration apparatus from Millipore (Millipore Corp. Bedfast, Mass., USA). Prior to this assay, the 0.2 ml aliquots were collected in small test tubes (10 x 75 mm) containing 1 ml 0.1 N NaOH 1 mM $HgCl_2$ and 100 µg/ml Tween 80. The test tubes were equilibrated in an ice bath. After a rotation shaking, these tubes could be stored at -20°C. After thawing, the tubes were mixed by rotation and ultrasonicated for 30 sec. Using Pasteur pipettes the test solution was filtered over Millipore membrane filters (HAWP 02500, 0.1 µm). The filtrate was collected in plastic tubes. Each reaction vial was rinsed with 0.5 ml 0.1 N NaOH containing 100 µg/ml Tween 80. After each rewash, the reaction vials were vigorously shaken on the rotation mixer. The last filtrates were even collected in the same vial. After drying of the Millipore membrane, the filter could be counted directly in 10 ml scintillation fluid. The filtrates were completely transferred into 10 ml scintillation solution acidified with 0.1 ml 1 N HCl and counted after shaking and equilibration.

6.3 Two-phase scintillation assay (TPSA): The TPSA was performed in plastic scintillation vials (Zinsser, Frankfurt) prefilled with 1 ml 0.05 N NaOH containing 1 mM $HgCl_2$. Aliquots of 0.2 ml which were withdrawn from each incubation vial were added to the scintillation vials containing 10 ml scintillation solution. During the alkaline measurement (pH 13), the amount of cholesterol present in the incubation medium can be counted. Thereafter, each scintillation vial was acidified with 0.1 ml 1 N HCl solution, shaken vigorously and counted after equilibration at 4°C in darkness for 24 hours. The amount of isocaproic acid per vial is calculated by differences between the impulse rate of both measurements. The impulse rate (cpm) after acidification represents the recovery.

6.4 Thin layer chromatography (TLC): In control tests, the enzymatic conversion of (4-^{14}C)-cholesterol to radioactive labeled pregnenolone, progesterone and other metabolites were analyzed by TLC (4). Progesterone could be visualized under UV by fluorescence. Cholesterol and pregnenolone could be stained with iodine. Further non-identified steroids were iodine-positive. All progesterone derivatives were also UV-positive. The localization of the radioactive metabolites on the plate was performed with a radioscanner (Berthold and Frieseke). The

localized radioactive steroids were scraped off the silica gel plates and counted directly in the scintillation vials.

7) Measurement of radioactivity:

A mixture of 5 g PPO, 80 g naphthalene, 330 ml xylene and 666 ml dioxane was used as scintillation solution (6). After addition of the radioactive samples the scintillation vials were vigorously shaken for 15 minutes and equilibrated for 24 hours at 4°C prior to scintillation counting. For scintillation measurements, a liquid scintillation counter from Nuclear Chicago, Mark II (Zinsser, Frankfurt, West Germany) operating at 84% efficiency for carbon-14 and at 60% efficiency for tritium was used. All scintillation counting was performed using a preset time of 10 minutes and preset count: 100,000 cpm.

RESULTS

Determination of the cholesterol side chain cleavage by means of the new test systems depends on the separation of (26-^{14}C)-cholesterol and (1-^{14}C)-isocaproic acid, which is formed equimolarly during the conversion of cholesterol to pregnenolone.

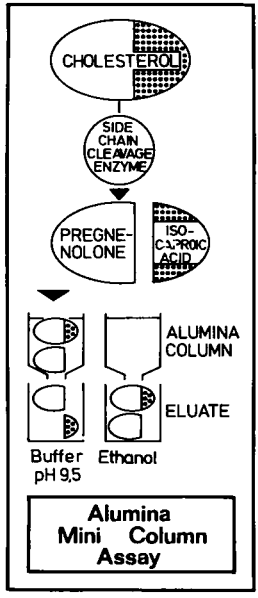

Fig. 1: Alumina Minicolum Assay

The radioactive side chain (labeled by dots) of (26-^{14}C)-cholesterol is split off by the cholesterol side-chain cleavage enzyme. Radioactive isocaproic acid and unlabeled pregnenolone is formed.
Cholesterol in an aqueous solution is adsorbed by the aluminium oxide of the mini-columns. In an alkaline medium, the smaller isocaproic acid sodium salt can percolate through the column and can be counted in the buffer eluate. By a second wash of the column with ethanol, the adsorbed cholesterol can be eluted and determined quantitatively.

1. Alumina minicolumn assay (AMCA):

The AMCA is a modification of a method described by Hochberg et al. (1) (Fig. 1). A contamination of the buffer eluate with cholesterol can be determined by means of a two-phase scintillation test of the buffer eluate. The flow sheet is presented in Fig. 2.

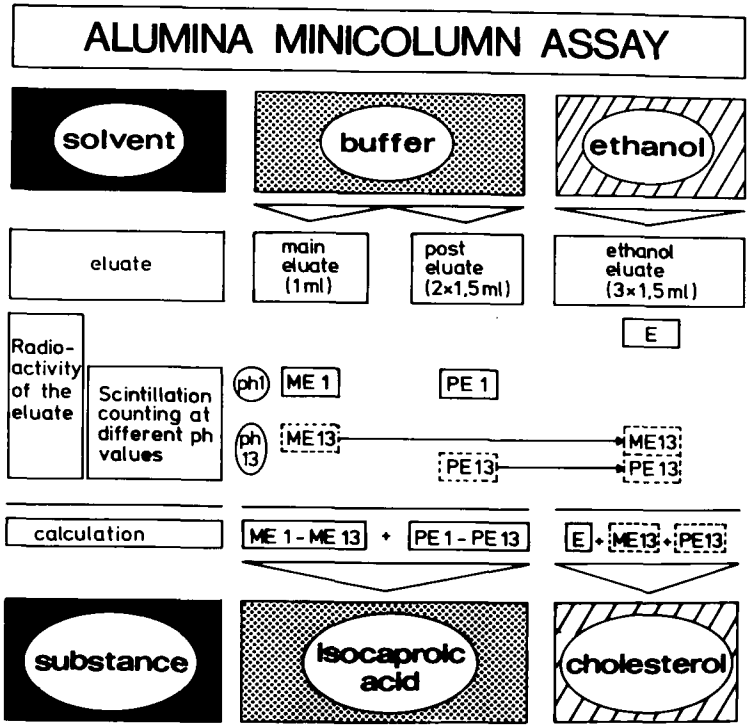

Fig. 2: Flow sheet for the calculation of the Alumina Minicolumn Assay

The AMCA depends on the different elution of isocaproic acid and cholesterol in two solvent systems. The smaller isocaproic acid sodium salt can pass the column in an aqueous phase. Cholesterol is adsorbed by the aluminium oxide and can be eluted by ethanol.

For the detection of small amounts of cholesterol in the buffer eluate (false positive = overestimation of isocaproic acid), a two-phase scintillation test is performed with the buffer eluate: scintillation measurements of the buffer eluate were performed at pH 1 and pH 13, followed by a subtraction of the impulse rates (cpm). The impulse difference correlates with the amount of isocaproic acid formed. The false positive amount of cholesterol in the buffer eluate is added to the cholesterol fraction in the ethanol eluate.

Abbreviations: Impulse rates (cpm) found by scintillation counting of the different eluates at pH 1 and pH 13:

ME_1 = buffer main eluate; pH 1 ME_{13} = buffer main eluate; pH 13
PE_1 = buffer posteluate; pH 1 PE_{13} = buffer posteluate; pH 13
E = ethanol eluate

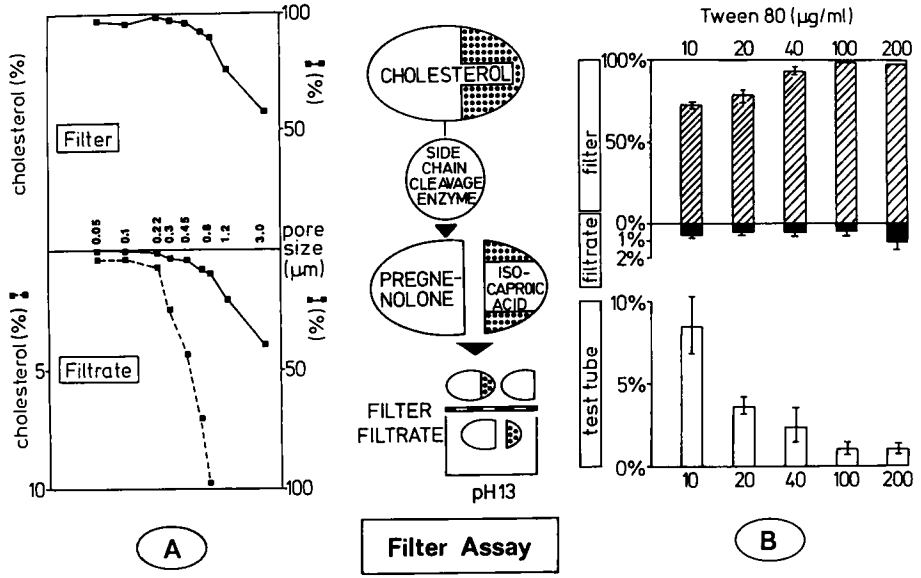

Fig. 3: Filter Assay

The radioactive side chain (labeled by dots) of the (26-^{14}C)-cholesterol is split off by the cholesterol side-chain cleavage enzyme. Radioactive isocaproic acid and unlabeled pregnenolone are formed.
The cholesterol molecule is retained by a Millepore membrane filter while the smaller isocaproic acid molecule appears in the filtrate. From the determination of the radioactivity of filter and filtrate the conversion rate can be calculated.

A: Influence of the pore size of the Millipore membrane filters on the permeability of the filter for cholesterol.

B: Influence of the Tween 80 concentration on the distribution of cholesterol on the filter (pore size 0.1/µm), in the filtrate and as a residue in the test tubes. ($\bar{x} \pm$ SD (n = 10))

2. Filter assay (FA):

The FA is based on the separation of cholesterol and isocaproic acid by means of a membrane filter (Fig. 3). With a pore size smaller than 0.1/µm, less than 1% cholesterol appears in the filtrate (Fig. 3A). The distribution of cholesterol on the filter (0.1/µm), in the filtrate and as a residue in the test tube at various Tween 80 concentrations is shown in Fig. 3B. In tests with 100/µg/ml Tween, less than 1% of cholesterol was found in the filtrate and the least amount of cholesterol was left behind in the test tube.

3. Two-phase scintillation assay (TPSA):

The TPSA is based on the pH-dependent distribution of isocaproic acid between an organic scintillation solution (upper layer) and an aqueous phase (lower layer) in a scintillation vial as demonstrated in Fig. 4.

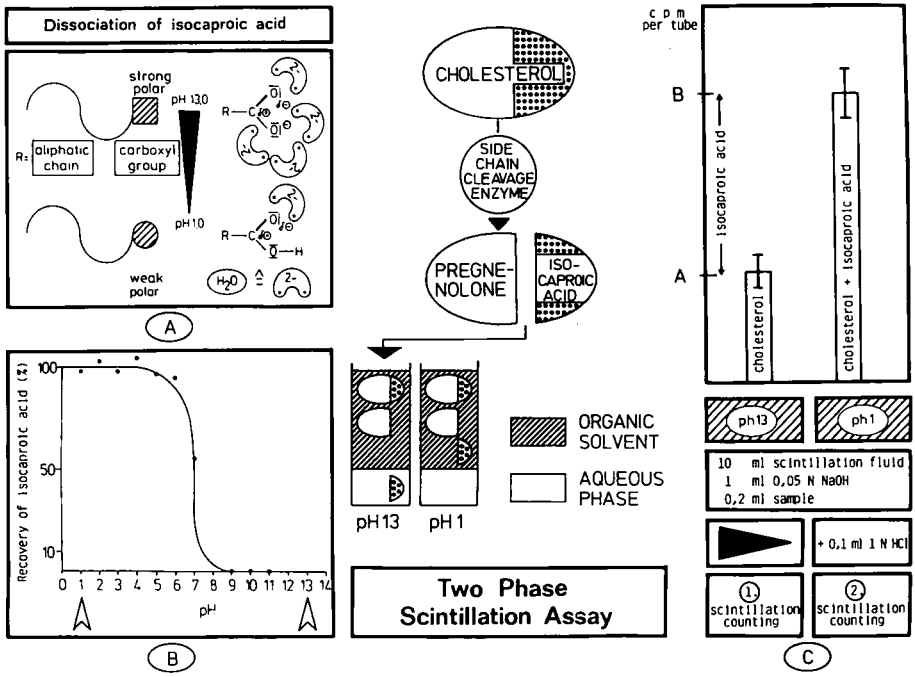

Fig. 4: Two-Phase Scintillation Assay (TPSA):

The radioactive side chain (labeled by dots) of (26-^{14}C)-cholesterol is split off by the cholesterol side-chain cleavage enzyme. Radioactive isocaproic acid and unlabeled pregnenolone is formed.

In the two-phase scintillation system consisting of an organic liquid scintillation solution (upper layer) and an aqueous phase (lower layer), there is a pH-dependent distribution of isocaproic acid, while cholesterol can be completely extracted by the organic phase independently of the pH value.

A: At pH 13, the carboxyl group of the isocaproic acid molecule can form a good hydration shell around the salt. At pH 1, the molecule is weakly polar, without a hydration shell, and behaves lipophilically.

B: A liquid scintillation counting of radioactive isocaproic acid is only possible when the test substance is dissolved in the scintillation solution. At pH 13, the isocaproic acid behaves polarly and cannot be extracted out of the aqueous phase. It therefore cannot be counted. After acidification of the aqueous phase to pH 1, there is a loss of the hydration shell. The molecule now behaves apolarly and can be extracted by the organic scintillation solution and counted to about 99%.

C: The TPSA depends on two scintillation measurements; the first is performed at pH 13 and the second at pH 1. Because the cholesterol molecule always stays in the scintillation phase irrrespective of the pH value, the amount of isocaproic acid can be calculated by acidification of the aqueous phase and subtraction of the impulse rates (cpm) at pH 1 and pH 13.

4. Criteria of reliability:

The sensitivity, accuracy, precision and specificity of the cholesterol side chain cleavage assays were analyzed both for the determination of single substances, cholesterol and isocaproic acid, and for the detection of cholesterol and isocaproic acid in the same test. With regard to in vitro studies of the cholesterol side chain cleavage enzyme with (26-^{14}C)-cholesterol as precursor, mixtures of a constant large amount of cholesterol (approx. 20 000 cpm/test) and increasing small amounts of isocaproic acid were analyzed.

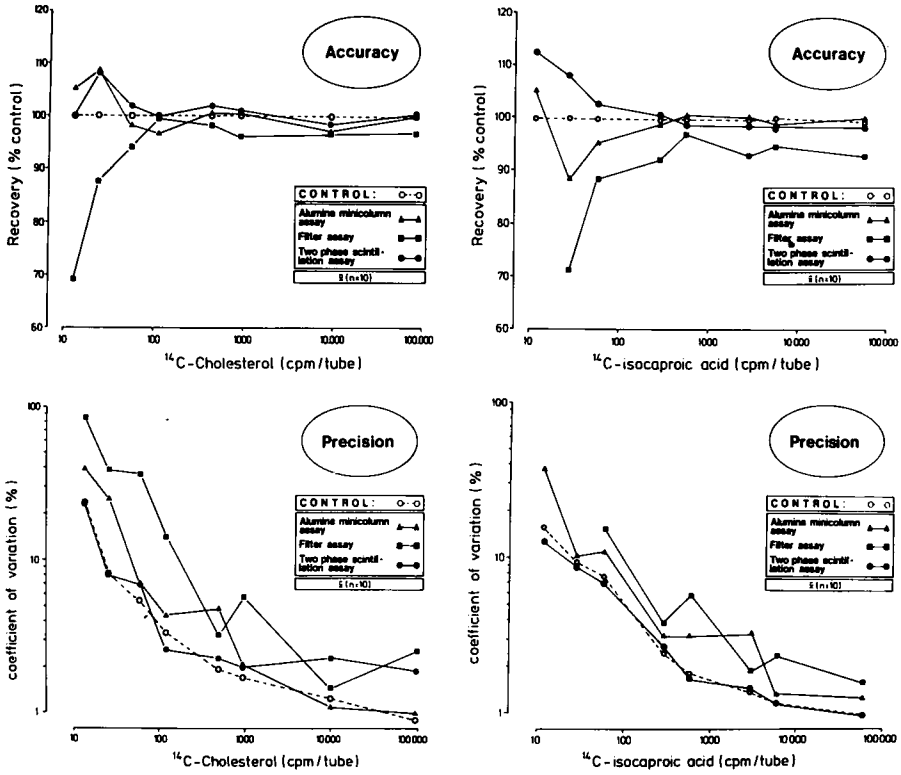

Fig. 5: Criteria of reliability of the cholesterol side chain cleavage assays: Determination of cholesterol and isocaproic acid in different tests.

The rate of recovery and the precision as demonstrated by the coefficient of variation are shown in relation to (26-^{14}C)-cholesterol and (1-^{14}C)-isocaproic acid concentrations. Test volume = 0.2 ml. $\bar{x}$ (n = 10).

4.1 Detection of single substances: cholesterol and isocaproic acid: Figure 5 shows the rate of recovery (accuracy) and precision of (4-^{14}C)-cholesterol and (1-^{14}C)-isocaproic acid determination as obtained with different cholesterol side chain cleavage tests.

Cholesterol: The sensitivity of all three tests ranged between 10 - 20 cpm cholesterol/test. The accuracy of the three test systems for the determination of cholesterol (100-85,000 cpm/test) ranged between 95 - 105%. For low cholesterol concentrations (10-100 cpm/test), the accuracy of the FA was not as good as for the AMCA and the TPSA. The best precision was achieved by the AMCA followed by the TPSA and the FA. For the determination of 1000 cpm cholesterol per test, a coefficient of variation (precision) of 2% was found for the AMCA and TPSA and of 6% for the FA.

Isocaproic acid: The sensitivity of AMCA and TPSA ranged between 10 - 20 cpm isocaproic acid per test. The sensitivity of the FA was found to be 25 - 50 cpm isocaproic acid per test. The accuracy of the AMCA and TPSA for the determination of isocaproic acid (100 - 50 000 cpm/test) ranged from 97 - 103%. The accuracy of the FA was lower as demonstrated by recovery rates of 90 - 95%. At low isocaproic acid concentrations (10 - 50 cpm/test), the TPSA showed a slight overestimation and the filter assay a loss of isocaproic acid as indicated by lower recovery rates. The best precision was obtained by the TPSA followed by the AMCA and FA.

4.2 Detection of cholesterol and isocaproic acid in mixtures: In incubations of the mitochondrial cholesterol side chain cleavage enzyme decreasing cholesterol levels and increasing concentrations of isocaproic acid were found. As a simplification of the test procedure, increasing amounts of ^{14}C-isocaproic acid (10-50,000 cpm/test) in combination with a constant concentration of (26-^{14}C)-cholesterol (20,870 cpm/test) usually recovered from aliquots (200 µl) taken from mitochondrial incubations were tested. By means of the three cholesterol side chain cleavage tests, the rate of recovery and accuracy of isocaproic acid and cholesterol assay in mixtures were determined as shown in Fig. 6.

With regard to studies with the isolated cholesterol side chain cleavage enzyme, the sensitivity of the different test systems is of interest only for isocaproic acid because during these experiments it is necessary to detect small amounts of isocaproic acid in presence of high cholesterol levels.

Sensitivity: The sensitivity (lowest concentrations of isocaproic acid which can be detected) is influenced by the cross reaction due to cholesterol. Therefore, the sensitivity is given as the concentration of isocaproic acid which can be determined in presence of a definite concentration of cholesterol (20,870 cpm/test).

As may be seen from Fig. 7, the AMCA and the FA allowed detection of very small amounts of isocaproic acid ranging from 20 -100 cpm/test, but in this range, there is a high overestimation due to a cholesterol contamination of the isocaproic acid fraction.

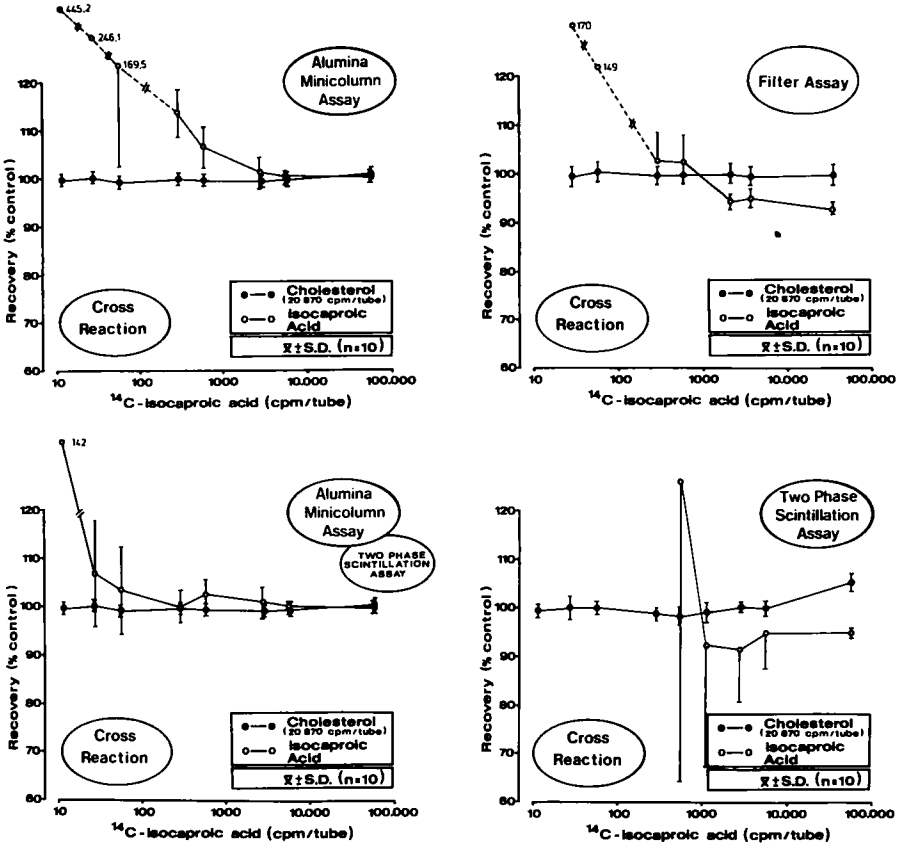

Fig. 6: Criteria of reliability of the cholesterol side chain cleavage tests: Determination of increasing concentrations of (1-^{14}C)-isocaproic acid in the presence of a constant concentration of (26-^{14}C)-cholesterol (20,870 cpm/test).

The cross reaction due to cholesterol or isocaproic acid respectively is demonstrated by means of an overestimation (false positive) of the respective substance. Test volume = 0.2 ml. $\bar{x} \pm$ SD (n = 10).

The sensitivity of the different test systems was compared on the basis of the amount of isocaproic acid which can be recovered with an accuracy between 90 and 110% and a coefficient of variation lower than 20%. According to this definition, the following sensitivities (cpm/test) of the test systems were found: combination of AMCA and the TPSA: 25, AMCA: 250, FA: 250 and TPSA: 2000.

Accuracy: The accuracy (rate of recovery) of the AMCA ranges between 97 -103% for 2000 - 50,000 cpm/test isocaproic acid. By combination of the AMCA and the TPSA, this concentration range is extended to 50 - 50,000 cpm/test. Using the FA, an overestimation of isocaproic acid for low concentrations (10 - 500 cpm/test) and a loss of isocaproic acid in a concentration range of 2000 to 50,000 cpm/test was found. For the TPSA, there is a high overestimation of isocaproic acid up to 2000 cpm/test and an accuracy of 90 - 95% in the range of 2000 - 50,000 cpm/test.

The accuracy of the cholesterol (20,870 cpm/test) determination is not influenced by isocaproic acid using the AMCA or the FA. In the presence of 5000 -50,000 cpm/test isocaproic acid, there is a slight increase of cholesterol recovery in the TPSA up to 105% of control values.

Precision: In all test systems, the precision for isocaproic acid increases with increasing amount of isocaproic acid in the test tube. The concentration of isocaproic acid necessary to obtain a coefficient of variation less than 10% was 250 cpm/test (AMCA), 50 cpm/test (combination of AMCA and TPSA) and 250 cpm/test (FA). For the TPSA, the standard deviation is high in a low concentration range of isocaproic acid as shown by the following coefficients of variation: 500 cpm/test: 60%, 2000 cpm/test: 30%, 5000 cpm/test: 10%, 50,000 cpm/test: 2%.

4.3 Comparison of the tests by cholesterol side chain cleavage in vitro performed with bovine adrenal mitochondria. The agreement of results obtained by cholesterol side chain cleavage assays with (26-^{14}C)-cholesterol and a chromatographic separation of (4-^{14}C)-cholesterol and radioactive metabolites after an incubation of bovine adrenal mitochondria is demonstrated in Fig. 7. As may be seen from this figure, there is a good agreement of all test systems. The TLC procedure for the separation of (4-^{14}C)-cholesterol and metabolites which are not identical with (4-^{14}C)-cholesterol is very time-consuming and difficult because of the presence of auto-oxidation products of cholesterol.

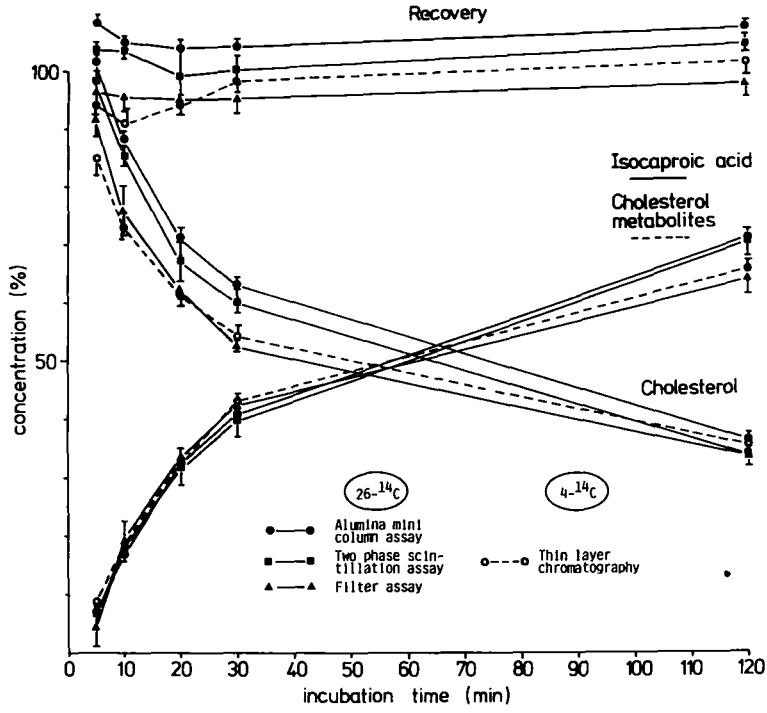

Fig. 7: Comparison of different cholesterol side chain cleavage tests:

Estimation of cholesterol side chain cleavage in bovine adrenal mitochondria by different test systems. Incubations were carried out using (26-^{14}C) and (4-^{14}C)-labeled cholesterol as substrate. (26-^{14}C)-cholesterol and (1-^{14}C)-isocaproic acid were determined by means of the alumina mini column assay, the filter assay and the two phase scintillation assay. (4-^{14}C)-cholesterol and radioactive labeled products e.g. pregnenolone and progesterone, were separated by means of thin layer chromatography. $\bar{x} \pm$ SD (n = 10)

4.4 Application for in vitro studies. For in vitro studies of the cholesterol side chain cleavage, it is necessary that the tests described above can be applied for the separation of isocaproic acid and cholesterol in presence of enzyme proteins, tissue homogenates and tissue explants.

Exogenous (1-^{14}C)-isocaproic acid could be recovered after eight hours of incubation by both AMCA or TPSA in a high yield (93 -95%) after incubation with human placental and bovine adrenal mitochondria. Similar results were obtained during incubations of human placental homogenates.

During studies of the side chain cleavage of (26-^{14}C)-cholesterol in

placental tissue explants, only very small amounts of both (26-^{14}C)-cholesterol in the tissue and radioactive isocaproic acid in the incubation medium could be detected. This excludes the application of these test systems for studies of cholesterol side chain cleavage in intact placental tissue. The application of the test systems for adrenal or ovarian tissue must be further investigated.

DISCUSSION

Isocaproic acid is formed equimolarly with pregnenolone by the side chain cleavage of cholesterol in bovine adrenal mitochondria (1) and in the human placenta (7). It is assumed that the first product which is formed after side chain cleavage is isocaproaldehyde, which is rapidly oxidized to isocaproic acid (8). During mitochondrial incubations, the concentration of the isocaproaldehyde is very low and can be only be detected radioisotopically after inhibition of its oxidation by large amounts of unlabeled isocaproaldehyde. For cholesterol side chain cleavage tests with mitochondria, the concentration of isocaproaldehyde may be disregarded.

Cleavage of the cholesterol side chain has been investigated so far in two different ways. One series of tests depends on the determination of non-radioactive pregnenolone arising on incubation with non-labeled cholesterol. Pregnenolone has been determined by means of colorimetry (9), gas liquid chromatography (10), radioimmunoassay (11) or by NADPH determination after enzymatic oxidation of pregnenolone to progesterone (12). The other tests are based on measuring the turnover of radioactive cholesterol, labeled at the sterol body (i.e. at the C-4 atom) (13) or at its side chain (C-26 atom). The radioactive cholesterol side chain (as isocaproic acid) has so far been determined by gas liquid chromatography (14), an extraction technique (15), steam distillation (16,17) and by alumina column chromatography (1). With the exception of the alumina mini column test (1), all these tests are very elaborate and only allow the processing of a few samples simultaneously.

In this paper, the development and application of two new and one modified test system for the determination of cholesterol side chain cleavage activity are described. All test systems depend on the separation of radioactively labeled isocaproic acid and radioactive cholesterol after side chain cleavage of (26-^{14}C)-cholesterol.

As a modification of the alumina mini column assay (AMCA) described by Hochberg et al. (1), the cholesterol concentration in test aliquots can be measured by elution of the columns with ethanol. In this way, no internal standard (e.g. tritiated water) is necessary. The determination of the cholesterol concentration in the incubation flask seems to be necessary because of an unstable aqueous solution of cholesterol in the aqueous phase (18-23).

A (false positive) overestimation of isocaproic acid by the AMCA was found due to the small amount of cholesterol which can percolate through the column in the buffer phase. By a combination of the AMC assay with the TPS assay of the buffer eluate, the overestimation of isocaproic acid can be reduced. The overestimation of isocaproic acid by cholesterol contamination of the buffer phase during the AMCA has not been described by other authors (1) because they only collect the first buffer eluate and do not add together the radioactivity found in separate buffer eluates during washing of the column. During experiments without cholesterol side chain cleavage enzyme, the first buffer eluate (1.0 ml) contained 70% of the total isocaproic acid (5,000 cpm/test) while the posteluate (1.5 ml) contained 28.6% to give a total recovery of 98.6% isocaproic acid. The cholesterol (20,000 cpm/test) overlap in the buffer main eluate was found to be 0.02 - 0.05% and in the posteluate 0.08 - 0.2%, giving a total cholesterol recovery in the buffer phase of 0.1 - 0.25%. The overestimation of isocaproic acid by a cholesterol contamination of the buffer phase is only important for the determination of small amounts of isocaproic acid. A low side chain cleavage of (26-^{14}C)-cholesterol is found in incubations with human placental mitochondria or homogenates due to the large amounts of endogenous unlabeled cholesterol. During these studies, the largest amount of isocaproic acid formed was found to be 1% to 10% (24). The amount of cholesterol in the buffer eluate of the alumina minicolumn can increase in presence of serum proteins added to test incubations to prevent the degradation of cholesterol side chain cleavage enzyme and of proteohormones used as test substances. Furthermore, this effect is important for studies of the mitochondrial cholesterol side chain cleavage enzyme when solubilizers (e.g. dimethylsulfoxide, Tween 80, ethanol, 1,2-propanediol) are necessary for dissolving apolar test substances in an aqueous incubation medium.

The filter assay (FA) depends on the separation of cholesterol and isocaproic acid by means of a Millipore membrane filter with a pore size smaller than an

exclusion value (0.1 /um). The retention of cholesterol by a Millipore membrane filter was described for the first time by Saad & Higuchi (18). Until now, no other group has published a test for the cholesterol side chain cleavage based on the separation of isocaproic acid and cholesterol by means of this membrane filter technique. At first, a micelle formation of Tween 80 and cholesterol was thought to be necessary for retention of cholesterol by the membrane filter, but during investigations of other steroids with higher aqueous solubility in absence of Tween 80, the retention of these steroids was found to be related to their polarity and structure (unpublished data). The filter assay can also be applied in other tests in which a small water-soluble and radioactively labeled group can pass the membrane filter after splitting off the molecule.

The two-phase scintillation assay (TPSA) used for the determination both of cholesterol side chain cleavage and radioisotopic measurement of other enzymatic reactions is described for the first time. Other authors have published tests depending on the extraction of isocaproic acid prior to a scintillation measurement (15,25) but have not described a pH-dependent extraction measurement of the radioactive substance in a two-layer (organic and aqueous phase) scintillation solution. The TPSA is very easy and rapid and therefore this test can be used for a large number of simultaneous determinations. Furthermore, the specificity of the AMCA and the FA can be improved by performing a TPSA with the eluate or filtrate respectively. The sensitivity of the TPSA is influenced by the overlap of the (26-^{14}C)-cholesterol and (1-^{14}C)-isocaproic acid determination which were counted in the same scintillation vial at different pH values. While cholesterol can be determined directly (pH 13), the amount of isocaproic acid is calculated by the impulse difference (cpm) as determined at pH 1 and pH 13. In this way, the sensitivity and precision of the isocaproic acid measurement is influenced by the statistical error of scintillation counting and the stability of the scintillation solution with regard to chemoluminescence and quench effects. The TPSA can be applied for all cholesterol side chain cleavage tests when the ratio of isocaproic acid is more than 5% of total cholesterol and when this amount of isocaproic acid is 2 - 5 times higher than of the blank values. The TPSA can be used not only for the determination of cholesterol side chain cleavage but also for reactions in which a polar group of a molecule is split off and the rest of the molecule now behaves apolarly and vice versa. The test is independent of the localization of the

radioactive label on the molecule, either on the residue which is split off or on the molecule which is left behind. The following enzymatic reactions are studied as an application of the two-phase scintillation assay: hydrolysis of steroid esters (e.g. steroid sulfates, steroid acetates, steroid glucuronides). Furthermore, the pH-independent partitition of tritiated water and (1,2-^{3}H)-androstenedione during aromatase studies has been analysed in a two-phase scintillation system (26).

Isocaproic acid is not metabolized by human placental and bovine adrenal mitochondria in vitro as could be demonstrated by a high recovery rate (93-95%) of exogenous isocaproic acid after 8 hours of incubation. The uptake of cholesterol by placental tissue in organ culture and therefore the formation and release of isocaproic acid is very low. Furthermore, an intracellular degradation of isocaproic acid is possible. In conclusion, cholesterol side chain cleavage tests, as described above, can be used for studies with isolated intact or broken mitochondria and with some reservations for tissue homogenates but not for organ culture experiments.

The test systems which have been described for the cholesterol side chain cleavage are easy to perform, are sensitive enough to follow enzyme activity, and results are obtained within a short time (27,28). The test systems have also proved useful for studies on the regulation of placental mitochondrial cholesterol side chain cleavage (24) and for pharmacological studies, e.g. of the inhibitory effect of aminoglutethimide on placental and bovine adrenal steroidogenesis (29).

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of Prof. C. Kaufmann, Cologne.

REFERENCES

1. Hochberg, R.B., van der Hoeven, T.A., Welch, M. and Lieberman, S. Biochemistry 13:603 (1974).
2. Burstein, S. and Gut, M. Recent progress in Hormone Research 27:303 (1971).
3. Burstein, S. and Gut, M. Steroids 28:115 (1976).
4. Simpson, E.R. and Boyd, G.S. Eur. J. Biochem. 2:275 (1967).
5. Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. J. Biol. Chem. 193:265 (1951).
6. Wagner, R.K. Hoppe-Seyler's Z. Physiol. Chem. 353:1235 (1972).
7. Gunasegaram, R., Peh, K.L., Chew, P.C.T., Karim, S.M.M. and Ratnam, S.S. Acta Endocr. (Kbh) 90:185 (1979).

8. Constantopoulos, G., Carpenter, A., Satoh, P.S. and Tchen, T.T. Biochemistry 5:1650 (1966).
9. Koritz, S.B. Biochim. Biophys. Acta 56:63 (1962).
10. Young, D.G. and Hall, P.F. Biochemistry 8:2987 (1969).
11. Bermudez, J.A., Doerr, P. and Lipsett, M.B. Steroids 16:505 (1970).
12. Simpson, E.R., Jefcoate, C.R., Brownie, A.C. and Boyd, G.S. Eur. J. Biochem. 28:442 (1972).
13. Halkerston, I.D.K., Eichhorn, J and Hechter, O. J. Biol. Chem. 236:374 (1961).
14. Burstein, S., Zamoscianyk, H., Co, N., Adelson, M., Prasad, D.S.M., Greenberg, A. and Gut, M. Biochim. Biophys. Acta 231:223 (1971).
15. Raggatt, P.R. and Whitehouse, M.W. Biochem. J. 101:819 (1966).
16. Kimura, T., Satoh, P.S. and Tchen, T.T., Anal. Biochem. 16:355 (1966).
17. Doering, C.H. and Clayton, R.B. Endocrinology 85:500 (1969)
18. Saad , H.Y. and Higuchi, W.I. J Pharm Sci 54:1205 (1965).
19. Fleischer, S. and Brierley, G. Biochemical and Biophysical Research Communications 5:367 (1961).
20. Ekwall, P., Baltscheffsky, H. and Mandell, L. Acta Chem. Scand. 15:1195 (1961).
21. Saunders, D.R. and Wells, M.A. Biochim. Biophys. Acta 176:828 (1969)
22. Kellaway, I.W. and Saunders, L. Biochim. Biophys. Acta 144:145 (1967).
23. Ekwall, P. and Mandell, L. Acta Chem. Scand. 15:1403 (1961).
24. Rabe, T. Weidenhammer, K. and Runnebaum, B. 2nd Innsbruck Winter Conference, Innsbruck 1981, submitted for publication.
25. Burstein, S. and Dorfman, R.I. J. Biol. Chem. 238:1656 (1963).
26. Rabe, T., Bierwirth, A.-M. and Runnebaum, B. 5th International Symposium of the Journal of Steroid Biochemistry, Mexico 1981, submitted for publication.
27. Rabe, D., Rabe, T. and Runnebaum, B. Acta Endocr. (Kbh), Suppl. 225 (1979)
28. Rabe, T., Rabe, D., Bierwirth, A.-M. and Runnebaum, B. 62nd Annual Meeting of the Endocrine Society, Washingtion 1980, Abstract No. 718.
29. Rabe, D., Rabe, T., Bierwirth, A.-M. and Runnebaum, B. 6th International Congress of Endocrinology, Melbourne, Australia 1980, Abstract No. 426.