PREGNENOLONE FORMATION FROM CHOLESTEROL IN BOVINE ADRENAL CORTEX MITOCHONDRIA: PROPOSAL OF A NEW MECHANISM

R. J. KRAAIPOEL, H. J. DEGENHART, J. G. LEFERINK*, V. VAN BEEK, H. DE LEEUW-BOON and H. K. A. VISSER

Department of Pediatrics, Erasmus University and Academic Hospital Rotterdam/Sophia Children's Hospital and Neonatal Unit, Gordelweg 160, Rotterdam

Received 21 November 1974

1. Introduction

Several models of cholesterol side-chain cleavage have been proposed, but none of them has found general acceptance [1–3]. The schemes favoured by most authors presently working in this field are (a) two consecutive hydroxylations of cholesterol at carbon atoms 20 and 22 (the classical scheme) and (b) a concerted attack of oxygen with the formation of 20α, 22R-di-OH cholesterol. The vicinal glycol is cleaved to yield pregnenolone and isocaproaldehyde.

It has been found that 20α-OH cholesterol, 20α, 22R-di-OH cholesterol and 22R-OH cholesterol are all effective precursors of pregnenolone in adrenal tissue [1,2]. Burstein and Gut [2,4,5] were able to isolate labelled 20α, 22R-di-OH cholesterol and 22R-OH cholesterol when labelled cholesterol was added to an acetone powder of bovine adrenal cortex mitochondria. Their calculations suggest that 20α, 22R-di-OH cholesterol is an intermediate of the cholesterol side-chain cleavage reaction. They also presented evidence that pregnenolone formation via the 'classical pathway' is insignificant and their results strongly indicate the existence of other (as yet unidentified) pathways.

From the experiments of Wilson [6] and Burstein [2] it is evident that a correlation exists between the rate of oxygen uptake and the rate of pregnenolone formation when 22R-OH cholesterol, 20α, 22R-di-OH cholesterol and 20α-OH cholesterol are added to adrenal cortex mitochondria. This correlation was used as a screening method for potential intermediates of the cholesterol side-chain cleavage reaction.

The results of our studies cannot be explained by the models proposed until now. Therefore a new model will be suggested in this paper. Preliminary results of this work have been presented elsewhere [7–9].

Nomenclature and abbreviations:
HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.
EGTA: Ethylene glycol-bis-(β-amino-ethylether)*N,N'*-tetraacetic acid.
Cyanoketone: 2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one.
Cholesterol: 5-cholesten-3β-ol.
20α-OH cholesterol: 5-cholesten-3β,20α-diol.
22R-OH cholesterol: (22R)-5-cholesten-3β,22-diol.
20α,22R-di-OH cholesterol: (22R)-5-cholesten-3β,20α,22-triol.
Δ^{20-22} cholesterol: 5,20(22)-cholestadien-3β-ol.
Δ^{17-20} cholesterol: 5,17(20)-cholestadien-3β-ol.
Δ^{20-21} cholesterol: 5,20(21)-cholestadien-3β-ol.
20,22-epoxycholesterol: 20,22-epoxy-5-cholesten-3β-ol.

* Laboratory for Toxicology, State University Utrecht. The Netherlands.

2. Materials and methods

Bovine adrenal cortex mitochondria were prepared according to standard procedures. Only mitochondria with a respiratory control ratio (succinate) ⩾ 3 were used.

Oxygen uptake was measured in a thermostated (37°C) vessel (vol 1.3 ml) equiped with a Clark electrode. The medium used contained: 200 mM sucrose, 20 mM KCl, 10 mM potassiumphosphate, 20 mM

North-Holland Publishing Company – Amsterdam

HEPES (pH 7.3), 1 mM EGTA, 1% bovine serum albumin (w/v). Further additions are mentioned in the legend to fig. 1.

$H_2{}^{18}O$ incorporation experiments were performed in a medium ($H_2{}^{18}O/H_2{}^{16}O$ = 1:2) containing 154 mM KCl, 11.5 mM NaCl, 50 mM nicotinamide, 20 mM HEPES (pH 7.3), 5 mM $CaCl_2$, 4 mM Na-azide, 1% bovine serum albumin (w/v) with a final vol of 5 ml. Bovine adrenal cortex mitochondria (stored under liquid nitrogen) were added to the $H_2{}^{18}O$ containing medium immediately after freeze-drying and kept for 30 min at 0°C (procedure *a*). Procedure *b* consisted of storing freeze-dried mitochondria in contact with the air during 30 min at 0°C, followed by addition to the $H_2{}^{18}O$ containing medium. These media containing mitochondria (15 mg protein) were preincubated at 37°C for 20 min. Additions were made to achieve a final concentration of 30 μM cyanoketone, 5 μM antimycin A, 200 μM 20α-OH cholesterol or 22R-OH cholesterol. The reactions were started by adding NADPH, glucose-6-phosphate and glucose-6-phosphate-dehydrogenase to give a final concentration of 0.1 mM, 3 mM and 0.6 U/ml respectively. Samples taken at 0, 10 and 20 min were extracted with ethylacetate, evaporated under nitrogen, persilylated with *N*-trimethylsilylimidazole by heating for 1 hr at 95°C in the presence of pyridine hydrochloride.

Gaschromatography: Analytical separations were carried out with a Hewlett-Packard 5700A instrument equiped with dual flame ionization detectors. The glass columns (2 m × 1.8 mm I.D.) were packed with 3% SP-2250 (Supelco) on 100–120 mesh Chromosorb WAW-DMCS H.P. The conditions were: injector

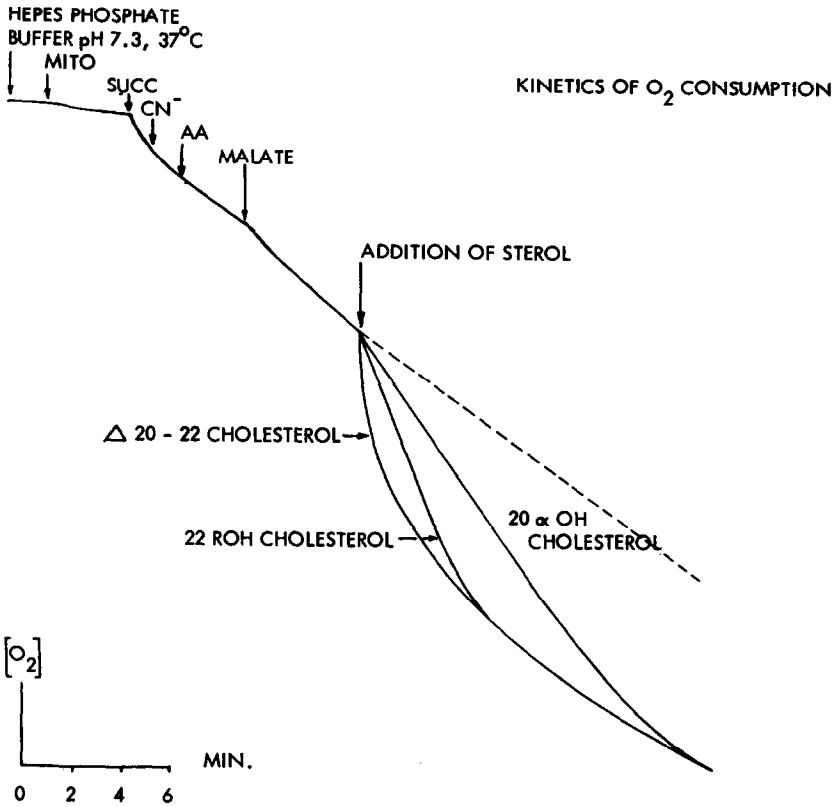


Fig. 1. Effect of the addition of sterols on the rate of oxygen uptake by bovine adrenal cortex mitochondria. Mitochondria (0.9 mg protein) were added to 1.25 ml medium (methods). Additions were made as indicated. Final concentrations were: 10 mM succinate, 1 mM potassium cyanide, 5 μM antimycin A, 10 mM malate, 20 μM 20α-OH cholesterol, 20 μM 22R-OH cholesterol, 20 μM Δ^{20-22} cholesterol. Sterols were added as their ethanolic solutions.

250°C, detector 300°C, nitrogen flow rate 30 ml/min. The oven was programmed from 236°C to 280°C at 2°C/min. Peak areas and retention times were measured with an electronic integrator (Infotronics CRS-100A).

Mass-spectrometry: The mass-spectra were recorded on a Finnigan 1015D-6000 GC-MS-computer system. The GLC-conditions were equivalent to those described above. Helium was used as a carrier-gas. The MS-conditions were: jetseparator at 250°C, source temperature 170°C, ionizing voltage 70 eV and ionizing current 500 μA.

Δ^{20-22} cholesterol was synthesized according to Sheikh and Djerassi [10]. Protein was estimated using the biuret method.

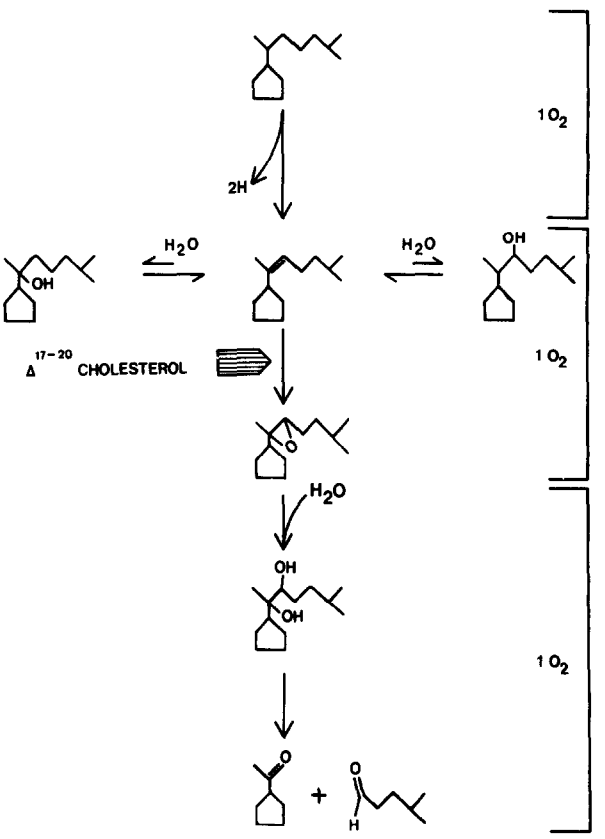
3. Results and discussion

The rate of oxygen uptake of intact bovine adrenal cortex mitochondria was dramatically increased by adding Δ^{20-22} cholesterol in comparison to the addition of 22R-OH cholesterol and 20α-OH cholesterol (fig. 1). All three substrates were converted into pregnenolone.

Dehydration of 20α-OH cholesterol produces a mixture of Δ^{17-20}, Δ^{20-22} and Δ^{20-21} cholesterol [10]. Δ^{17-20} cholesterol proved to be a very efficient inhibitor of the cholesterol side-chain cleavage system. Contamination by this inhibitor might be the reason why Δ^{20-22} cholesterol was reported earlier as a very poor substrate [2].

Combination of our results with the evidence available in the literature, allowed us to propose a new scheme (fig. 2), whereby cholesterol is converted into pregnenolone and isocaproaldehyde via Δ^{20-22} cholesterol, 20,22-epoxycholesterol, 20α, 22R-di-OH cholesterol. 20α-OH cholesterol and 22R-OH cholesterol will lose water to form Δ^{20-22} cholesterol.

According to this proposal $H_2{}^{18}O$ should be incorporated in 20α,22R-di-OH cholesterol and the ^{18}O-label must be found in pregnenolone or isocaproaldehyde. To check this hypothesis 1000 nmol 20α-OH cholesterol and an equal amount 22R-OH cholesterol were incubated in parallel in a $H_2{}^{18}O$ containing medium ($H_2{}^{18}O/H_2{}^{16}O$ = 1:2) in the presence of freeze-dried bovine adrenal cortex mitochondria, supported by a NADPH generating system



(methods). After 20 min of incubation at 37°C (procedure *a*) 20α-OH cholesterol was partially converted; 330 nmol pregnenolone were formed. 22R-OH cholesterol was totally converted; 890 nmol pregnenolone were produced. Samples from these $H_2{}^{18}O$ incubations were extracted, trimethylsilylated and analyzed by GC-MS. Their mass-spectra were compared with that of synthetic pregnenolone and with those obtained in identical experiments in presence of unlabelled water (fig.3). The relative abundance of the M+2 peak of the ^{18}O incorporated pregnenolone shows an increase of 170% over the pregnenolone-standard (inaccuracy of the measurement: less than 10%). It could be concluded that water from the medium was incorporated in 20% of the product originating from 20α-OH cholesterol. No ^{18}O could be detected in the synthesized pregnenolone (standard), pregnenolone

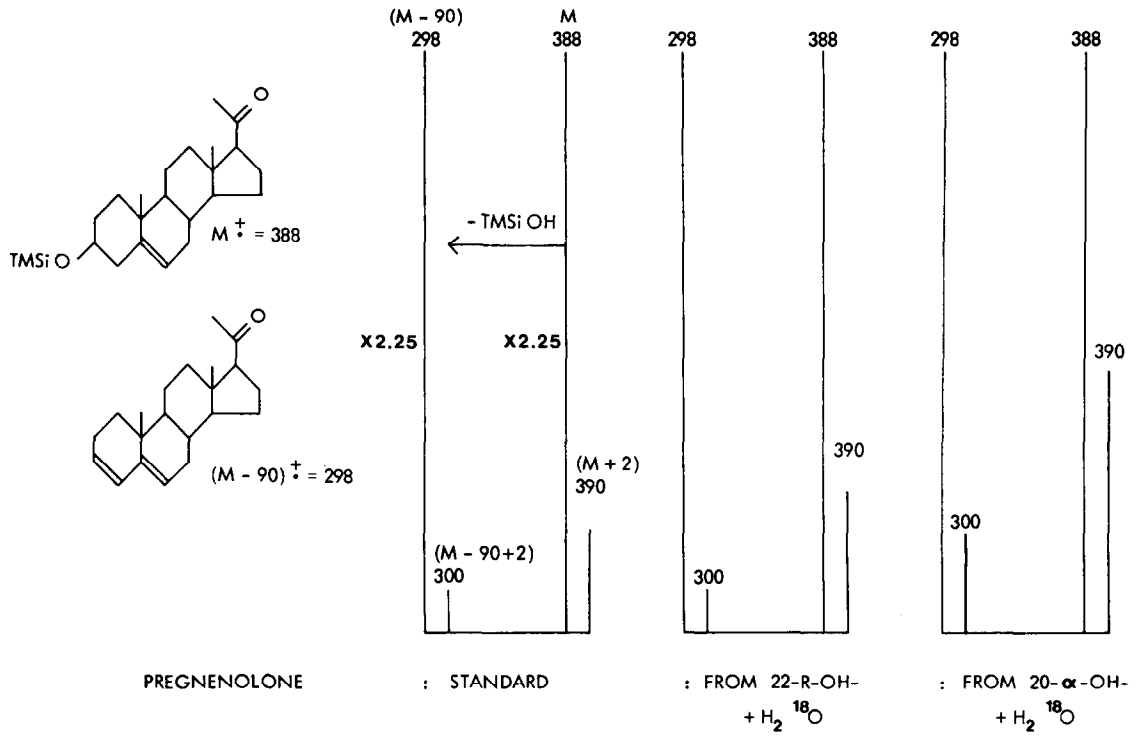


Fig. 3. The measured relative abundances of M-90+2 and M+2 of the pregnenolone-standard are in accord to the calculated natural abundances. The mass-spectrum of pregnenolone originating from 20α-OH cholesterol shows an incorporation of ^{18}O that can be seen from the marked increase in the relative abundances of M-90+2 and M+2. No incorporation was demonstrated in pregnenolone formed from 22R-OH cholesterol.

originating from 22R-OH cholesterol ($H_2{}^{18}O$) and pregnenolone from the control experiments. We could not demonstrate exchange of ^{18}O between the substrates 20α-OH cholesterol, 22R-OH cholesterol and $H_2{}^{18}O$. The low percentage of incorporation may be explained by the amount of water still present in the freeze-dried mitochondria and the water produced during the reaction (fig. 2). Pregnenolone originating from endogenous substrates contributes $\leqslant 5\%$ of the total pregnenolone production, causing only insignificant isotope dilution.

In measuring ^{18}O incorporation in 20α,22R-di-OH cholesterol, reaction conditions had to be changed (procedure *b*; methods) in order to isolate sufficient material for mass spectrometric identification. We found that 22R-OH cholesterol was partially converted and that 180 nmol pregnenolone and 157 nmol 20α,22R-di-OH cholesterol were formed. No detectable incorporation of ^{18}O could be found in pregnenolone; however ^{18}O was incorporated in 20α, 22R-di-OH cholesterol. Analysis of the mass-spectrum showed that ^{18}O was attached to the carbon-22 atom of 20α,22R-di-OH cholesterol. 20α-OH cholesterol incubated according to procedure *b* was partially converted; 70 nmol ^{18}O-incorporated pregnenolone were formed. The results are summarized in fig. 4.

Our results complement those of Takemoto et al. [11] and Nakano et al. [12] who performed incorporation studies with molecular oxygen ($^{18}O_2$). Incorporated ^{18}O in pregnenolone originating from cholesterol and 22R-OH cholesterol was found to be attached to the C-20 atom. In the presence of $^{18}O_2$ neither 20α-OH cholesterol nor 20α,22R-di-OH cholesterol was converted into pregnenolone containing labelled oxygen.

An explanation for the two different ways of the reaction of 20,22-epoxycholesterol with water (fig. 4) might be found by considering the aconitase reaction.

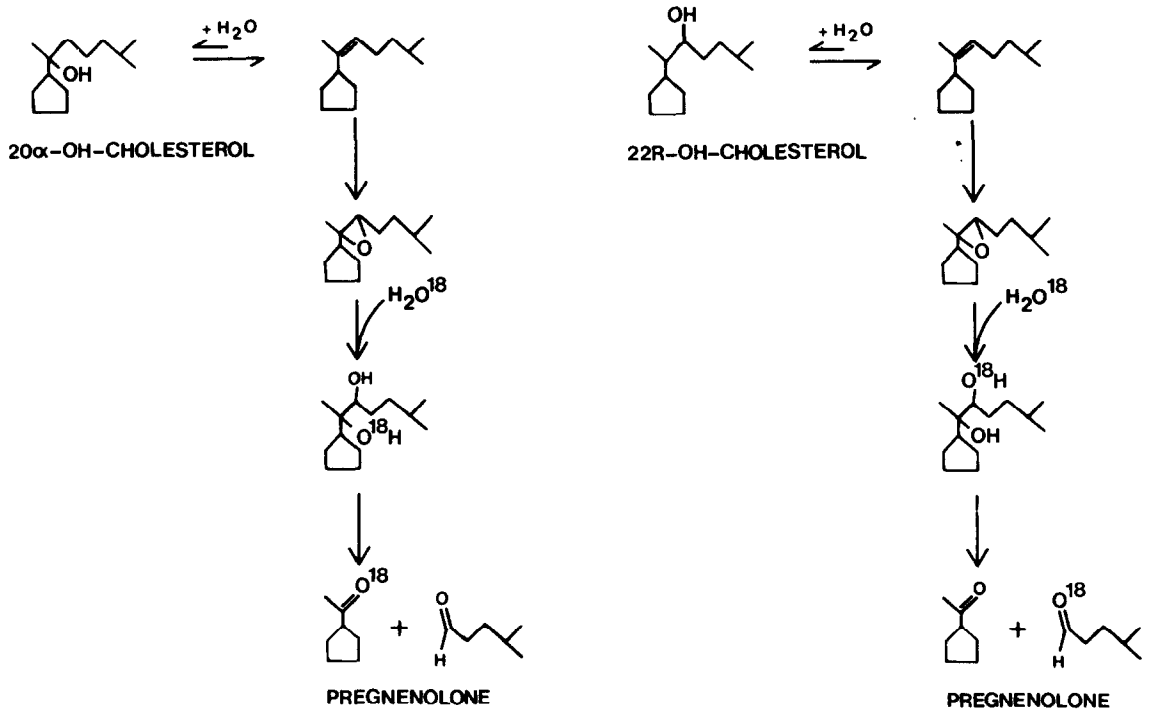


Fig. 4. Incorporation of ^{18}O after incubation of 20α-OH cholesterol and 22R-OH cholesterol with freeze-dried bovine adrenal cortex mitochondria supported by a NADPH-generating system in the presence of $H_2{}^{18}O$ (methods).

We postulate that Δ^{20-22} cholesterol originating from 20α-OH cholesterol is attached to the enzyme surface in a stereochemically different way than Δ^{20-22} cholesterol originating from 22R-OH cholesterol. This situation would be analogous to the aconitase reaction, where cis-aconitate formed by dehydration of citrate and cis-aconitate formed by dehydration of threo-D_s-isocitrate are bound in a stereochemically different way [13].

The proposed scheme (fig. 2) is also in accord with the stoichiometry of the reactions with respect to O_2 and NADPH, as reported by Shikita and Hall [14], and Kraaipoel et al. [7,8]. The conversion of 1 mol of cholesterol into Δ^{20-22} cholesterol would use at least $\frac{1}{2}$ mol of O_2 to form H_2O with the removed hydrogen. Activation of cytochrome P-450 [15,16] with 1 mol NADPH + H^+ probably needs another $\frac{1}{2}$ mol O_2. Since the conversion of both 20α-OH and 22R-OH cholesterol to pregnenolone and isocaproaldehyde requires 2 mol O_2 and 2 mol NADPH + H^+ per mol substrate, and the conversion of 20α,22R-di-OH cholesterol needs 1 mol O_2 plus 1 mol NADPH + H^+, it can be concluded that 1 mol O_2 plus 1 mol NADPH + H^+ are necessary for the conversion of Δ^{20-22} cholesterol into 20,22-epoxycholesterol.

20α,22R-di-OH cholesterol and 20,22-epoxycholesterol could be isolated from reaction media [20]. The high conversion rate of Δ^{20-22} cholesterol as an intermediate has hitherto prevented its isolation.

Virtually all the data found in the literature concerning cholesterol side-chain cleavage fit nicely into this new scheme [2,17,18]. Its consequences for the study of inborn errors in pregnenolone biosynthesis [19] will be discussed elsewhere [20].

Acknowledgements

Cyanoketone was a gift from Sterling-Winthrop. This work was supported by a grant from the Netherlands' Foundation for Fundamental Medical Research.

References

[1] Sulimovici, S. I. and Boyd, G. S. (1969) Vitamins and Hormones 27, 199–234.
[2] Burstein, S. and Gut, M. (1971) Recent Progress in Hormone Research 27, 303–349.
[3] Luttrell, B., Hochberg, R. B., Dixon, W. R., McDonald, P. D. and Lieberman, S. (1972) J. Biol. Chem. 247, 1462–1472.
[4] Burstein, S., Zamoscianyk, H., Kimball, H. L., Chaudhuri, N. K. and Gut, M. (1970) Steroids 15, 13–60.
[5] Burstein, S., Kimball, H. L. and Gut, M. (1970) Steroids 15, 809–857.
[6] Wilson, L. D. (1972) Biochemistry 11, 3696–3701.
[7] Kraaipoel, R. J., Degenhart, H. J., Visser, H. K. A., van Beek, V. J. M. B., de Leeuw, P. J. and Leferink, J. G. (1974) Acta Paediat. Scand. 63, 333.
[8] Kraaipoel, R. J., Degenhart, H. J., Leferink, J. G., van Beek, V., de Leeuw-Boon, H. and Visser, H. K. A. (1974) J. Steroid. Biochem. 5, 308.
[9] Degenhart, H. J. (1974) in: Clinical Biochemistry, Principles and Methods (Curtius, H. Ch. and Roth, M., eds.) pp. 668–672, W. de Gruyter, Berlin, New York.
[10] Sheikh, Y. M. and Djerassi, C. (1973) J. Org. Chem. 38, 3545–3554.
[11] Takemoto, C., Nakano, N., Sato, H. and Tamaoki, B. (1968) Biochim. Biophys. Acta 152, 749–757.
[12] Nakano, H., Inano, H., Sato, H., Shikita, M., Tamaoki, B. (1967) Biochim. Biophys. Acta 137, 335–346.
[13] Mahler, H. R. and Cordes, E. H. (1971) in: Biological Chemistry, 2nd Edn. pp. 609–610, Harper and Row, New York.
[14] Shikita, M. and Hall, P. F. (1974) Proc. Natl. Acad. Sci. 71, 1441–1445.
[15] Shikita, M. and Hall, P. F. (1973) J. Biol. Chem. 248, 5598–5604.
[16] Shikita, M. and Hall, P. F. (1973) J. Biol. Chem. 248, 5605–5609.
[17] Hochberg, R. B., McDonald, P. D., Feldman, M. and Lieberman, S. (1974) J. Biol. Chem. 249, 1277–1285.
[18] Tait, A. D. (1972) Biochem. J. 128, 467–470.
[19] Degenhart, H. J., Visser, H. K. A., Boon, H. and O'Doherty, N. J. (1972) Acta Endocr. (Kbh) 71, 512–518.
[20] Degenhart, H. J. and Kraaipoel, R. J.: In preparation.