The Stimulation of Pregnenolone Synthesis in the Large Particles from Rat Adrenals by Some Agents Which Cause Mitochondrial Swelling*

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Pregnenolone (3β-hydroxy-5-pregnen-3-one) synthesis in the large particles (mitochondrial fraction) from rat adrenal is stimulated by calcium, fatty acids, sodium lauryl sulfate, and proteolytic enzymes. These agents also promote swelling of the large particles. ATP, which inhibits the swelling produced by these agents, also inhibits their ability to stimulate pregnenolone synthesis. The stimulation of pregnenolone synthesis is reflected in an increase in corticoid output when the stimulatory agents are tested with whole homogenates. The data are interpreted to indicate that changes in mitochondrial permeability may have a role in the regulation of the synthesis of pregnenolone from endogenous precursors.

A role for calcium in steroidogenesis in the rat adrenal gland has been indicated by a variety of observations. Birmingham *et al.* (1953) have shown that calcium is required for the action of ACTH[1] *in vitro*, and this observation was confirmed and extended by Péron and Koritz (1958). In addition, it has been shown that calcium stimulates corticoid synthesis from endogenous precursors in whole homogenates of rat adrenal (Koritz and Péron, 1959); the site of this stimulation was to be found in the large particles of the homogenate (Péron and Koritz, 1960). Halkerston *et al.* (1961), using radioactive cholesterol, found that the enzymes for cholesterol side-chain cleavage occur in the mitochondria of beef adrenal cortex, and Koritz (1962), using the large particles from the rat adrenal, demonstrated that, in this fraction, pregnenolone synthesis from endogenous precursors can be stimulated by calcium. This would also indicate that the mitochondrial component of the large particle fraction is responsible for the synthesis of pregnenolone.

One of the *in vitro* effects which calcium is known to have on liver mitochondria is to induce swelling (Lehninger, 1959). Since, as indicated above, the biosynthesis of pregnenolone in the adrenal is associated with the large subcellular particles of this organ, the possibility presented itself that the stimulation by calcium may be associated with the swelling phenomenon. On this basis, various agents which have been shown to induce *in vitro* swelling in liver mitochondria have been tested with the large particles from rat adrenal as well as with rat adrenal whole homogenates in order to examine their effects on pregnenolone and corticoid synthesis, respectively. In addition, the previously observed stimulation of corticoid synthesis by proteolytic enzymes (Koritz and Péron, 1959) has been extended to this system.

In this report it is shown that agents which are apparently capable of affecting mitochondrial permeability are able to promote the stimulation of pregnenolone synthesis from endogenous precursors in the large particles from rat adrenal homogenates and that this was reflected in a corresponding increase in corticoid production in whole homogenates.

Experimental

Adrenal glands from male Sprague-Dawley rats weighing 200–300 g were homogenized in 0.154 M KCl and the homogenate fractionated as described elsewhere (Péron and Koritz, 1960). It should be emphasized that these glands were not preincubated as had been the case in previous studies. Before use, pellet-2 (the large particles) was resuspended in 0.154 M KCl on a basis of 90 mg wet weight of the original tissue per ml of final homogenate. When whole homogenates were used they were prepared on the basis of 1 g wet weight of tissue per 15 ml of homogenate. Pregnenolone production was measured by the procedure reported by Koritz (1962) with 1.5 ml of dichloromethane used in the final extraction. In experiments with whole homogenates, appropriate aliquots of the incubation mixture were extracted with dichloromethane; corticoid values obtained by a blue tetrazolium reaction (Elliot *et al.*, 1954) are reported as micrograms of corticosterone. Protein was measured by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. In pellet-2 preparations the protein usually ranged from 0.65–0.9 mg per 0.3 ml.

Since the glucose-6-phosphate dehydrogenase used in these experiments was obtained from several sources, an amount was used which was shown to give, in the presence of excess glucose-6-phosphate, essentially complete reduction of the amount of TPN used during the first two minutes of incubation. Fatty acids were solubilized with 0.3 N KOH and hot water immediately before use, and were neutralized with 1 N HCl. The sulfhydryl enzymes, papain, ficin, and bromelin were activated with 0.001 M Versene and 0.005 M cysteine (Kimmel and Smith, 1954), and the controls for incubations involving these enzymes contain Versene and cysteine. To determine proteolytic activity in various enzyme preparations, azocoll, which is an insoluble compound consisting of a degraded collagen coupled to an azo dye, was used as a substrate, and the amount of dye which was released into solution upon incubation in 0.1 M phosphate buffer, *p*H 7.5, for 15 minutes at 37° was measured optically at 580 mμ.[2]

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[1] Abbreviations used in this paper: ACTH, adrenocorticotropic hormone; ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-monophosphate; CTP, cytosine-5′-triphosphate; GTP, guanosine-5′-triphosphate; TPN^+, oxidized triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; versene, ethylenedinitrilotetraacetic acid.

[2] Information for this assay was supplied by the California Corporation for Biochemical Research, Los Angeles.

Mitochondrial swelling was measured by the change in optical density at 520 mμ as reported by Cleland (1952). The samples were incubated at 37°, and readings were made at 5-minute intervals on a Beckman DU spectrophotometer. An amount of the pellet-2 suspension was used which gave an initial absorbancy of approximately 0.600. The pellet-2 suspension was found to swell slightly on storage at 0° for three to four hours, but this did not seriously effect its response to the swelling stimuli. In these experiments 0.20–0.25 ml of pellet-2 was used in a final volume of 2.5 ml.

Materials

Disodium glucose-6-phosphate, TPN^+, TPNH, ATP, ADP, AMP, CTP, GTP, *Vipera russelli* venom, and *Crotalus adamanteus* venom were obtained from the Sigma Chemical Company.

Erepsin and myristic acid were obtained from Mann Research Laboratories, Inc.

Pronase, a bacterial proteolytic enzyme, bromelin, fibrinolysin, rennin, enterokinase, porcine pancreatic lipase, oleic acid, palmitic acid, azocoll, and stearic acid were purchased from the California Biochemical Corporation.

Phospholipase C, collagenase, papain (2 × crystallized), ficin (2 × crystallized), α-chymotrypsin (3 × crystallized), trypsin (2 × crystallized), carboxypeptidase A (3 × crystallized), elastase (60% $(NH_4)_2SO_4$ suspension), lysozyme (2 × crystallized), hyaluronidase, acid phosphatase, alkaline phosphatase, spleen phosphodiesterase, snake venom phosphodiesterase, and wheat germ lipase were supplied by the Worthington Biochemical Corporation.

Nagarse, a bacterial proteolytic enzyme, was obtained from Teikoku Chemical Ind. Co., Ltd., Japan.

The glucose-6-phosphate dehydrogenase used was prepared by the following: California Biochemical Corporation, Sigma Chemical Company, and Nutritional Biochemicals Corporation.

Sodium lauryl sulfate was obtained from the Fisher Scientific Company.

Results

The data of Table I demonstrate that pregnenolone synthesis in the pellet-2 preparation is stimulated by a variety of substances. This synthesis has been found to be essentially linear for more than 60 minutes (Koritz, 1962), and these results were also obtained in the presence of several stimulatory agents. In agreement with the results of Halkerston *et al.* (1961), no pregnenolone synthesis could be found in the absence of the TPNH-generating system. Calcium has been shown previously to stimulate pregnenolone synthesis in this system (Koritz, 1962), and the fatty acids and detergents were used because they, in common with calcium, have the ability to swell liver mitochondria *in vitro* (Lehninger and Remmert, 1959; Lehninger, 1959; Hunter and Ford, 1955). The fatty acids were all effective in stimulating the synthesis of pregnenolone from endogenous precursor, as was true for the detergent, sodium lauryl sulfate. Sodium deoxycholate and sodium taurocholate were ineffective at similar concentrations, and Tween 80 had no effect at 1×10^{-4} mg to 1 mg per beaker. The TPNH-generating system (TPN, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) could be replaced by TPNH and identical results obtained.

The proteolytic enzyme, ficin, has been shown to stimulate corticoid production in whole homogenates of rat adrenal (Koritz and Péron, 1959) and for this reason it was tested in the present system along with other enzymes. The five proteolytic enzymes listed in Table I have the best stimulatory activity. Other enzyme preparations which have much less activity are collagenase (0.1 mg to 1 mg per beaker), elastase (0.2 mg to 1.9 mg per beaker), *Vipera russelli* venom (0.01 mg to 1 mg per beaker), phospholipase C (1 mg to 5 mg per beaker), alkaline phosphatase (0.1 mg to 1 mg per beaker), pancreatic lipase (1 mg to 5 mg per beaker), and wheat germ lipase (0.1 mg to 1 mg per beaker). The venom has been shown to contain proteolytic activity (Grasset and Schwartz, 1955). Proteolytic activity was demonstrated in the four last-named preparations using the azocoll procedure. However, not all enzymes with proteolytic activity were stimulatory, since trypsin, α-chymotrypsin, carboxypeptidase A, nagarse, erepsin, rennin, and enterokinase either gave no stimulation or were inhibitory. It is of interest that Koritz and Péron (1959) also found that trypsin, chymotrypsin, and carboxypeptidase had no effect on corticoid synthesis in whole homogenates of rat adrenal. In addition, the following enzyme preparations either had no effect or were inhibitory: lysozyme, hyaluronidase, acid phosphatase, snake venom phosphodiesterase, spleen phosphodiesterase, and *Crotalus adamanteus* venom. In the present system, all of the latter enzymes were tested at a concentration of 1×10^{-4} mg to 1 mg per beaker, except for nagarse, *Crotalus adamanteus* venom, and enterokinase (1×10^{-3} mg to 1 mg per beaker), and spleen phosphodiesterase (5×10^{-4} units to 5 units per beaker).

The fatty acids, calcium, and lauryl sulfate, as mentioned previously, are all effective swelling agents of liver mitochondria *in vitro*. Since ATP has been shown to reverse the swelling of liver mitochondria *in vitro*, its effect on the stimulation of pregnenolone synthesis by these substances was tested. The results of these experiments are given in Table II, and it is seen that the enhancement of pregnenolone synthesis by the different stimulatory agents is prevented to varying degrees by ATP. The nucleotide inhibition of the calcium stimulation was tested in greater detail, and inspection of Table II reveals that at equimolar

Table I
Stimulation of Pregnenolone Synthesis by Various Agents[a]

Agent	Final Concentration	Pregnenolone, μg/mg protein	
		Control	+ Agent
Calcium	0.011 M	0.80	3.51
Calcium	0.011 M	0.43	6.74
Stearic acid	0.0001 M	0.61	1.82
Palmitic acid	0.0001 M	0.97	2.66
Myristic acid	0.0001 M	0.97	3.45
Oleic acid	0.0001 M	0.66	3.22
Na lauryl sulfate	0.0001 M	0.46	2.41
Pronase	1.0 mg/ml	0.63	3.21
Bromelin	0.5 mg/ml	1.79	3.94
Fibrinolysin	0.005 mg/ml	0.96	2.68
Papain	0.03 mg/ml	1.25	3.52
Ficin	0.7 mg/ml	1.13	3.03

[a] The incubation medium contained 0.3 ml of pellet-2, 90 μmoles phosphite buffer (pH 6.2), 1.6 mg sodium TPN^+, 2.1 mg glucose-6-phosphate, glucose-6-phosphate dehydrogenase, the stimulating agents, and 0.154 M KCl to a final volume of 2.0 ml. Incubation was for 1.0 hour at 37 ± 1° in air. The data represent several experiments. With calcium, greater variation was observed than with the other stimulatory agents, and the data given represent upper and lower limits of stimulation.

TABLE II
THE INHIBITION BY NUCLEOTIDES OF THE STIMULATION OF PREGNENOLONE SYNTHESIS[a]

Stimulatory Agent	Nucleotide	Final Concentration of Nucleotide	Pregnenolone, μg/mg protein			
			Control	+ Stimulatory Agent	+ Stimulatory Agent and Nucleotide	% Inhibition by Nucleotide
Calcium	ATP	4.6×10^{-3} M	0.55	2.82	0.39	100
Calcium	ADP	5.0×10^{-3} M	0.46	4.73	0.78	92
Calcium	AMP	5.0×10^{-3} M	0.61	5.10	4.86	5
Calcium	CTP	5.0×10^{-3} M	0.43	6.74	4.56	31
Calcium	GTP	5.0×10^{-3} M	0.43	6.74	6.48	4
Na lauryl sulfate	ATP	3.7×10^{-2} M	0.73	2.80	0.67	100
Myristic acid	ATP	4.6×10^{-2} M	0.97	3.45	1.60	75
Palmitic acid	ATP	4.6×10^{-2} M	0.97	2.66	1.20	87
Oleic acid	ATP	3.7×10^{-2} M	0.67	2.24	1.18	68
Pronase	ATP	2.0×10^{-2} M	0.63	3.21	1.57	63

[a] The contents of the incubation medium are the same as in Table I, except for the addition of nucleotides. Incubation was carried out at 37 ± 1° in air for 60 minutes in all cases, except for oleic acid which was for 30 minutes. The final concentration of pronase was 1.0 mg/ml, calcium was at 11 mM, and all other stimulatory agents were at a final concentration of 0.1 mM.

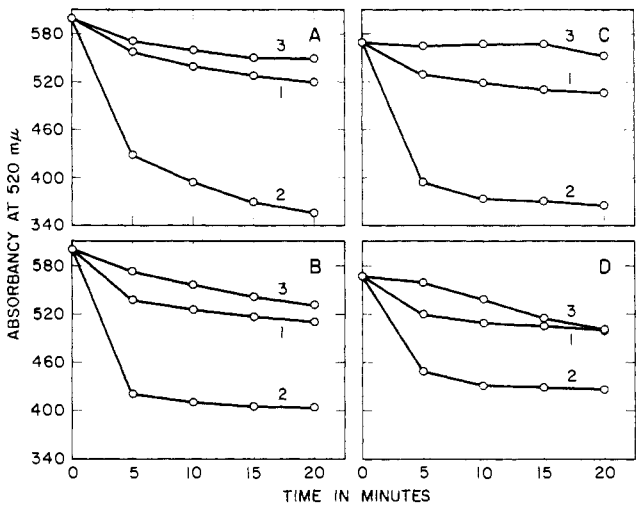

FIG. 1.—The ATP inhibition of swelling induced by several agents. In all cases curve 1 is the control and curve 2 represents the action of the swelling agents alone. These agents are: A, pronase, 2 mg; B, Na lauryl sulfate, 1×10^{-4} M; C, palmitic acid, 1×10^{-4} M; D, myristic acid, 1×10^{-4} M. Curve 3 represents the swelling agent + ATP at a concentration of 2.4×10^{-2} M in A, and 3.6×10^{-2} M in the remaining experiments. The test medium consisted of 90 μmoles phosphite buffer, *p*H 6.2, 0.2 ml pellet-2 suspension, and 0.154 M KCl to a volume of 2.5 ml. All reactants were present at the initiation of the experiments.

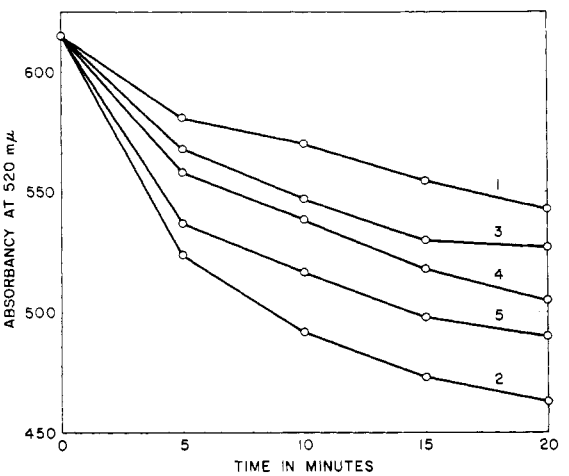

FIG. 2.—The induction of swelling by calcium and its inhibition by adenine nucleotides. The curves are as follows: 1, control; 2, calcium; 3, calcium + ATP; 4, calcium + ADP; 5, calcium + AMP. Swelling was induced by 11 mM calcium in a medium containing 110 μmoles phosphite buffer, *p*H 6.2, 0.25 ml pellet-2 suspension, and 0.154 M KCl to a volume of 2.5 ml. The nucleotides, which were present at the beginning of the experiment, were at a concentration of 5 mM.

concentrations ATP and ADP are the most effective nucleotides, whereas AMP and GTP have little effect. The inhibition of calcium stimulation by ATP at the concentration used did not require the addition of magnesium. In these experiments the nucleotide and the stimulatory agent were both present at the start of the incubation. Lower concentrations of ATP (0.92×10^{-3} M) inhibited the calcium stimulation 90–95% at 30 minutes, but only 40–50% at 60 minutes. With oleic acid, a substantial inhibition could be achieved only at shorter periods of incubation.

The relation between stimulation of pregnenolone synthesis and swelling may be seen from the data presented in Figures 1 and 2. Calcium, palmitic acid, myristic acid, pronase, and sodium lauryl sulfate all induce swelling of the pellet-2 preparation. The concentrations used here correspond to the concentrations which give maximal stimulation of pregnenolone synthesis. It is also evident that ATP concentrations which inhibit the stimulation of pregnenolone synthesis also inhibit the swelling induced by these agents. The effect of adenine nucleotides on swelling was examined in greater detail for the case of calcium stimulation (Fig. 2). The order of effectiveness was ATP > ADP > AMP, although there was only a small difference between ADP and ATP corresponding to their effects on pregnenolone synthesis (Table II). Magnesium was tested in the presence of ATP and had no significant effect.

Although the data presented up to now indicate a qualitative correlation between swelling and pregnenolone synthesis, this correlation does not hold quantitatively. For example, myristic acid promotes pregnenolone synthesis more than palmitic acid, but, as Figure 1 shows, the latter induces greater swelling. An extreme example is oleic acid, which produces a good stimulation of pregnenolone synthesis but causes little swelling in the pellet-2 preparation. This can be seen in Figure 3, where a direct comparison with calcium and palmitic acid is presented. However, as pointed

TABLE III
THE STIMULATION OF CORTICOID SYNTHESIS IN WHOLE HOMOGENATES BY AGENTS WHICH STIMULATE PREGNENOLONE SYNTHESIS IN PELLET-2[a]

Addition	Final Concentration	Corticoid Synthesis (μg/mg protein)
—	—	0.93
Calcium	1.1×10^{-2} M	4.46
Na lauryl sulfate	3.0×10^{-4} M	1.91
Myristic acid	3.0×10^{-4} M	1.89
Oleic acid	3.0×10^{-4} M	2.53
Palmitic acid	6.0×10^{-4} M	2.48
Pronase	0.005 mg/ml	2.18

[a] The incubation medium contained 0.4 ml homogenate, 40 μmoles of $NaHCO_3$, 1.6 mg $NaTPN^+$, 2.1 mg glucose-6-phosphate, the stimulating agents, and 0.154 M KCl to 2.0 ml. Incubation was for 1.0 hour at 37 ± 1° under 95% O_2-5% CO_2.

out previously (Table II) ATP partially reversed the stimulation of pregnenolone synthesis by oleic acid.

Table III demonstrates that all agents which are capable of stimulating the synthesis of pregnenolone from endogenous precursor(s) in pellet-2 also stimulate the formation of corticoids in whole homogenates of rat adrenal fortified with TPN and glucose-6-phosphate. Again calcium is the most potent agent in stimulating this synthesis. The orders of effectiveness of these various agents in promoting the synthesis of pregnenolone and corticoids do not necessarily correspond. This is not surprising, since the conditions for the two assays differ. For example, there is a difference in *p*H, and there may be binding of fatty acids in whole homogenates by proteins not present in the pellet-2 preparation.

DISCUSSION

The data presented suggest that changes in mitochondrial permeability may be of consequence in the control of pregnenolone synthesis in the adrenal, although the following discussion must be tempered by the realization that mitochondrial swelling agents often operate by indirect and complex mechanisms. In every case tested in which mitochondrial swelling was demonstrated there was a concomitant increase in the synthesis of pregnenolone. Similarly, when the mitochondrial swelling was inhibited by ATP, the stimulation of pregnenolone synthesis was curtailed. However, there was not a quantitative relationship between the stimulation of pregnenolone synthesis and the extent of swelling. Thus, the data reveal that palmitate is a more effective swelling agent than myristate or Ca^{2+}, but the latter stimulate pregnenolone synthesis to a greater extent than does palmitate. An extreme example is seen in the case of oleate, which causes relatively little swelling at the concentration at which it acts as an effective stimulator of pregnenolone synthesis. These results suggest that swelling by itself is not the critical phenomenon in the stimulation of pregnenolone synthesis, but rather that an underlying modification of the mitochondrial membrane, which may have swelling as one of its consequences, is of prime importance. This is emphasized by the observation that in a soluble preparation obtained from an acetone powder prepared from beef adrenal cortex mitochondria (Halkerston *et al.*, 1961) the conversion of cholesterol to pregnenolone is not stimulated by calcium.[3] Such a modification of the mitochondrial membrane may function, in the present system, to facilitate entry of exogenous TPNH or, alternately, since only endogenous precursor(s) of pregnenolone are involved, to permit pregnenolone formed to leave the mitochondria. Experiments designed to differentiate between these possibilities have not provided data amenable to an unequivocal interpretation. We have observed that when pregnenolone synthesis is measured as a function of TPNH concentration at different stimulatory levels of calcium the stimulatory response first appeared at the same concentration of TPNH, regardless of the calcium concentration. Tentatively, this may be interpreted to indicate that TPNH entry is not involved, since, if it were, one might expect that the effect of a higher calcium concentration would be apparent at a lower TPNH concentration than that of a lower calcium concentration.

[3] Unpublished observations by the authors.

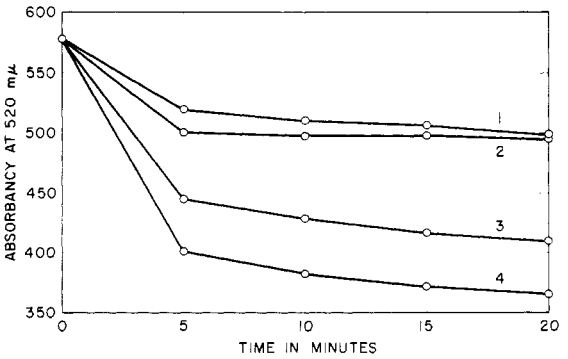

FIG. 3.—Comparison of swelling induced by oleate, calcium, and palmitate. Swelling was induced by 0.1 mM oleate (curve 2), 11 mM calcium (curve 3), and 0.1 mM palmitate (curve 4), in a medium consisting of 90 μmoles phosphite buffer, *p*H 6.2, 0.2 ml pellet-2 preparation, and 0.154 M KCl to a volume of 2.5 ml. Curve 1, the control, represents the spontaneous swelling in the P-2 preparation.

An explanation involving the removal of pregnenolone from the mitochondria is made attractive by the recent report of Koritz and Hall (1964). They have found in a soluble system from beef adrenal cortex that the transformation of cholesterol to pregnenolone is inhibited by pregnenolone at the first step of this sequence of reactions, i.e., the hydroxylation of cholesterol to 20α-hydroxycholesterol. Thus, any factor which would promote the removal of pregnenolone would alleviate the inhibition and stimulate the synthesis of pregnenolone. Stone and Hechter (1954) have reported that stimulation of steroidogenesis by ACTH occurs between cholesterol and pregnenolone, and it is thus tempting to speculate that this stimulation may occur by an alteration of the mitochondrial membrane. It is of interest that ACTH administration, *in vivo*, has been reported to result in an increase in the number of adrenal cortical mitochondria with discontinuities in their membranes and with vesicles protruding at the mitochondrial surface (Lever, 1956; Sabatini *et al.*, 1962).

The action of the bacterial protease, pronase, on pellet-2 merits further discussion. Initially, it was considered that the stimulation of pregnenolone observed in the presence of this enzyme was a result of an alteration of the mitochondrial surface due to proteolytic action. However, the observation that pronase could promote swelling in the large particles, and that this swelling could be prevented by ATP, suggests that the protease causes the release of a substance from the mitochondria which actively promotes the swelling, and that it is the action of this substance which is antagonized by ATP. This is analogous to the release

of U factor by Ca^{2+} (Wojtczak and Lehninger, 1961). It can also be postulated that the proteolytic enzyme is directly inhibited by ATP, but the above explanation seems more plausible. Although the other proteolytic enzymes which stimulate pregnenolone synthesis were not examined in this respect, it is possible that some proteolytic enzymes constitute a new class of mitochondrial swelling agents.

It has been shown in previous studies that Ca^{2+} stimulates corticoid synthesis from endogenous precursors in rat adrenal homogenates (Koritz and Péron, 1959). This synthesis requires the presence of the large particles (pellet-2) and the supernatant, and of the two fractions pellet-2 is rate limiting (Péron and Koritz, 1960). In addition, Ca^{2+} has been shown to stimulate the synthesis of pregnenolone in isolated pellet-2 (Koritz, 1962). In view of these findings it was to be expected that any agent capable of stimulating the synthesis of pregnenolone in pellet-2 would also stimulate the overall synthesis of corticoids in adrenal whole homogenates. This exception is borne out by the data of Table III, where it is seen that all agents tested which stimulate pregnenolone synthesis also stimulate corticoid synthesis in the whole homogenate.

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Antimetabolites of Mevalonic Acid. II. Inhibition of Ergosterol Synthesis in Yeast*

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The synthesis of three groups of new mevalonic acid analogs is described: 5-deoxy analogs, 5-methyl ethers, and aromatic analogs. Bioassay of these compounds and several previously prepared analogs showed that most of them inhibited growth of the mevalonic acid–requiring organism *Lactobacillus acidophilus*, and that certain members of each group caused partial inhibition of biosynthesis of ergosterol by yeast. The most active inhibitor of sterol synthesis was 3-hydroxy-3-methyl-5-phenyl-4-pentenoic acid, which caused 50% inhibition of ergosterol synthesis in yeast at a concentration (0.3 mg/ml) which did not inhibit growth. This compound, however, did not inhibit cholesterol synthesis when fed to growing mice.

Since the identification of mevalonic acid (3,5-dihydroxy-3-methylvaleric acid; MVA) as a key intermediate in the biosynthesis of sterols (Tavormina *et al.*, 1956), a number of investigators have sought to inhibit cholesterol biosynthesis by the use of structural analogs of MVA as possible antimetabolites. Mentzer *et al.* (1956) early found that 3-methyl-3-pentenoic acid would lower the liver cholesterol level of rats, while Gey *et al.* (1957) and Weiss *et al.* (1961) found that a number of compounds related structurally to MVA would inhibit the synthesis of cholesterol from MVA in rat liver homogenates. Several fluorinated analogs of MVA were investigated by Singer *et al.* (1959) and Kirschner *et al.* (1961); some compounds were found with high anti-MVA activity in enzyme systems. Daeniker and Druey (1960) found that certain derivatives of 5-thio MVA had a hypocholesteremic effect in rats. None of these assays, however, has provided accurate information about the effect of the analogs on sterol synthesis in an intact organism; the examination of serum cholesterol levels may give information primarily on transport, rather than synthesis, while *in vitro* enzyme assays may well not be subject to feedback and other control mechanisms which operate in the intact animal.

Intact organism assays are available for examination of the effects of MVA analogs on sterol synthesis and on other functions of this metabolite. Microbiological assays with sensitive strains of lactobacilli were used by Tamura *et al.* (1958) and by Stewart and Woolley (1959) to detect anti-MVA activity of a number of MVA analogs. Although the use of these organisms does not provide information on inhibition of sterol synthesis (since they do not synthesize sterols), the simplicity and great sensitivity of the *Lactobacillus* assay made it very useful during early stages of the investigation. Other intact organism assays which do involve sterol biosynthesis have been used (Stewart and

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