known hemeprotein. The possibility may be suggested that in mitochondria, the incorporation of iron into the porphyrin–protein complex occurs by way of a hemeprotein intermediate which transfers the synthesized heme to form the hemeprotein found in the 0.35–0.50 fraction of the cholate extract. The hypothesis of heme transfer has been repeatedly proposed by several authors.

The nature of the 0.50–0.75 fraction and the product of ^{59}Fe incorporation is now being investigated.

Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo, Tokyo (Japan)

SHIGEKI MINAKAMI
YOSHIMASA YONEYAMA
HARUHISA YOSHIKAWA

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Further studies on enzymic adrenal 11-β-hydroxylation

11-β-Hydroxylation of the steroid nucleus, occurring in adrenal mitochondria, has been under study for a considerable period in several laboratories[1–4], during which it has become apparent that molecular oxygen provides the hydroxyl oxygen and that reduced triphosphopyridine nucleotide (TPNH) is a necessary reactant.

More recently[5] we have found that two heat-labile enzyme fractions, in addition to TPNH, were required to catalyse the reaction. One of these was extracted from acetone powder of adrenal mitochondria with an aqueous digitonin solution. The second could be prepared from other tissues as well as the adrenal, *e.g.*, liver, spleen, plasma.

In the experiments reported here, deoxycorticosterone, the substrate, was 11-hydroxylated to form corticosterone. The extent of the reaction was assayed fluorimetrically[5].

Because the digitonin used in our earlier adrenal preparation rendered its further purification difficult, the adrenal mitochondrial acetone powder was extracted instead with 10 vol. distilled water (H_2O extract) and the extracted residue was extracted a second time, using 10 vol. 0.15 *M* KCl (KCl extract). The results, some of which differed from those reported earlier[5], are shown in Table I.

Both adrenal extracts (*i.e.*, H_2O and KCl) were apparently necessary in order for the hydroxylation to proceed. Further, it was found that the rate was greatly enhanced if an aqueous extract of rabbit-liver acetone powder were added to the two adrenal fractions. This liver extract could be heated to 100° for several minutes without losing its ability to stimulate the reaction. Several adrenal enzyme preparations were obtained which were completely inactive without the addition of a boiled liver extract. A heat-stable hydroxylation cofactor could also be obtained from acetone powders of human placenta and bovine testis, and from crude adrenal homogenates. It thus appeared that not only were two enzymes and TPNH needed for 11-hydroxylation, but that

TABLE I

COFACTOR REQUIREMENT FOR 11-β-HYDROXYLATION

The reaction mixtures contained 0.04 μmole TPNH, 0.029 μmole deoxycorticosterone, 10 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.4, enzymes, and other additions in a vol. of 0.57 ml. Incubated 20 min at 37° in air.

	Corticosterone found (mμmoles)
H_2O extract	1.33
KCl extract	0
H_2O extract + liver extract	1.66
KCl extract + liver extract	0.42
H_2O extract + KCl extract	0.84
H_2O extract + KCl extract + liver extract	6.72
H_2O extract + KCl extract + boiled liver extract	5.72

there was an additional cofactor requirement as well. None of the following, tested at 10^{-3} *M*, was able to replace the unknown cofactor: ascorbic acid, glutathione, nicotinamide, nicotinamide riboside, fumarate, citrate, additional TPNH, a TPNH-generating system (glucose-6-phosphate, glucose-6-phosphate dehydrogenase and TPN), H_2O_2 or a H_2O_2-generating system (glucose and glucose oxidase), folic acid, tetrahydrofolic acid or phenylalanine-hydroxylating cofactor[6]. (The latter two were gifts of Dr. SEYMOUR KAUFMAN of the National Institute of Mental Health.)

The active material, obtained from liver, could not withstand ashing and was not adsorbed by charcoal at pH 2, nor could it be extracted into 10 vol. of ether on methylene chloride from the aqueous phase at acid, neutral, or alkaline pH. The acidic character of the cofactor was suggested by its retention on Amberlite IRA-400 chloride or phosphate (from which it could be recovered) and its electrophoretic mobility on paper at pH 6. Additional purification of this coenzyme is in progress.

The two adrenal enzymes were purified further: the H_2O extract by adsorption on and elution from alumina Cγ gel, and the KCl extract by treatment with calcium phosphate gel, which could be followed by ethanol precipitation. The results of these experiments are shown in Table II. It was found that, although either of the more purified proteins was active when tested with the other unpurified fraction, the combination of both purified enzymes (in the presence of TPNH and the heat-stable liver cofactor) had lost its hydroxylating ability. Neither heated extracts of the crude water or KCl fractions, nor an unheated extract of the liver acetone powder, when added to

TABLE II

EFFECTIVE PURIFICATION OF THE ENZYMES REQUIRED FOR HYDROXYLATION

The reaction mixtures contained 0.1 μmole TPNH, 0.029 μmole deoxycorticosterone, 10 μmoles tris(hydroxymethyl)aminomethane buffer, pH 7.4, boiled liver extract (except in the last experiment), in a vol. of 0.7 ml. Incubated 20 min at 37° in air.

	Corticosterone found (mμmoles)
Crude H_2O extract + crude KCl extract	6.19
Purified H_2O extract + crude KCl extract	5.55
Crude H_2O extract + purified KCl extract	6.98
Purified H_2O extract + purified KCl extract	0.89
Purified H_2O extract + purified KCl extract + 3rd digitonin extract	7.30
Purified H_2O extract + purified KCl extract + boiled digitonin extract	0.54
Purified H_2O extract + purified KCl extract + crude liver extract	0

the other components, could restore the activity. This was interpreted to mean that a *third* enzyme, contaminating both the crude H_2O and KCl extracts but not present in the purified fractions, was also required for 11-β-hydroxylation. This third, heat-labile fraction could be best obtained by extraction of the adrenal mitochondrial acetone powder a third time (*i.e.*, after the initial extractions with H_2O and KCl) with aqueous 0.5% digitonin.

Because of the numerous components required for 11-β-hydroxylation, *i.e.*, 3 distinct enzymes, TPNH, molecular oxygen, and an unidentified heat-stable coenzyme, the reaction mechanism must be quite complicated. In support of this, the complete system frequently demonstrated a 5–10 min lag period before the formation of 11-hydroxysteroid began, which suggested the formation of at least one intermediate in the overall process.

It should be noted that in our previously reported results[5], an initial digitonin extract of the adrenal mitochondrial acetone powder showed a requirement for a heat-labile, non-dialyzable (presumably enzymic) fraction derived from liver or other organs. In the present system, there is no liver *enzyme* required, but instead a heat-stable coenzyme, also obtainable from liver.

The difference between these two hydroxylating systems is currently under study.

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service, Bethesda, Md. (U.S.A.)

GORDON M. TOMKINS
JEAN F. CURRAN
PATRICIA J. MICHAEL

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