into two additional components by chromatography on gel-cellulose in the presence of 4 M urea (3). One of the latter components (6.3 S) was the flavoprotein, dihydrodiol dehydrogenase (2). The other component (21.2 S) was colorless, contained essentially all of the protein-bound lipoic acid of the complex, and exhibited dihydrolipoic transacetylase activity (Table I). This component is tentatively designated lipoic dehydrogenase-transacetylase.

All three components, carboxylase, lipoic dehydrogenase-transacetylase, and flavoprotein, were required to reconstitute the CoA- and DPN-linked oxidation of pyruvate (Table II). Examination of a mixture of the three components (ratio of 1.3:1:0.5 by weight) in a Spinco model E ultracentrifuge revealed the virtual absence of peaks corresponding to the individual components and, instead, a major peak (52.8 S) with which the boundary of the yellow color of the flavoprotein was associated (Fig. 1). The mixture was centrifuged for 2 hours at 173,000 × g in a Spinco model L ultracentrifuge and the composition and enzymatic activities of the yellow pellet were determined (Table I). The results indicate that the three isolated components combined to produce a large unit resembling the original complex. Other sedimentation studies indicated that the carboxylase and the flavoprotein did not combine with each other, but that each of these components did combine with the lipoic dehydrogenase-transacetylase. Experiments are in progress to determine the stoichiometry of the reconstitution of the complex.

### Table II

<table>
<thead>
<tr>
<th>Component</th>
<th>Acetyl phosphate produced (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylase</td>
<td>0</td>
</tr>
<tr>
<td>Lipoic dehydrogenase-transacetylase</td>
<td>0</td>
</tr>
<tr>
<td>Flavoprotein</td>
<td>0</td>
</tr>
<tr>
<td>Carboxylase + flavoprotein</td>
<td>0</td>
</tr>
<tr>
<td>Carboxylase + lipoic dehydrogenase-transacetylase</td>
<td>0.08</td>
</tr>
<tr>
<td>Lipoic dehydrogenase-transacetylase + flavoprotein</td>
<td>0.25</td>
</tr>
<tr>
<td>Carboxylase + lipoic dehydrogenase-transacetylase + flavoprotein</td>
<td>2.12</td>
</tr>
</tbody>
</table>

* The amounts of protein per ml were: carboxylase, 2 μg; lipoic dehydrogenase-transacetylase, 2 μg; flavoprotein, 3 μg. Other components and conditions were as described previously (1).

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**An Action of Gonadotropin In Vitro**

**Norman R. Mason,† John M. Marsh, and Kenneth Savard†**

*From the Endocrine Laboratory, University of Miami School of Medicine, Miami, Florida*

(Received for publication, March 27, 1961)

The synthesis in *vivo* of progesterone by luteal tissue from the sow (1) has recently been reported. We wish at this time to report the stimulation in *vitro* of this biosynthesis in cow corpus luteum by means of gonadotropin preparations of pituitary and urinary origin.

Bovine corpus luteum was obtained from nonpregnant cows at the abattoir and immediately chilled. The tissues were sliced by means of a Stadie-Riggs hand microtome, and the slices from a single corpus luteum were distributed among the incubation vessels of each experiment. By this means, the effects of added substances were assessed on tissue from the same corpus luteum. Incubations were carried out at 37°C in a Dubnoff metabolic shaking incubator for 2 or 3 hours and were terminated by rapidly freezing the contents of the vessels. A trace amount of progesterone-4-C<sup>14</sup> of high specific activity was added to each vessel, and tissue and medium homogenized and extracted according to the method of Short (2). The residue from each extract was subjected to a 5-transfer countercurrent distribution between petroleum ether (boiling range, 65–110°C) and methanol-water (9:1). The fraction containing the polar lipids was purified by chromatography on silica gel, eluting with benzene containing increasing concentrations of ethyl acetate. The progesterone fraction was chromatographed on paper in the ligroin-propylene glycol system (3). The progesterone zone (ultraviolet-absorbing area coincident with radioactive tracer) was eluted and the amount of the steroid measured quantitatively.

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**REFERENCES**

by its absorption at 240 m/ in methanol, with use of the correction suggested by Allen (4). The recovery of the tracer progesterone-4-C\textsuperscript{14} permitted correction for mechanical losses. This extensive procedure was necessary to remove the large amount of substances which interfered with the spectroscopic measurement of progesterone. In this regard, the method of Legault-Demare et al. (5) for the measurement of progesterone in incubated corpus luteum, based on the ultraviolet absorption of crude methylene dichloride extracts, does not achieve this purification and in our hands is totally unreliable.

Our results on bovine tissues, summarized in Table I, fully confirm those of Duncan et al. (1) in demonstrating the ready formation of progesterone in surviving corpus luteum slices. The results also reveal the considerable variability in progesterone content (Column 2) and in the biosynthetic capacity of luteal tissues (Column 3), attributed by these authors to the age of the corpus luteum (1). True de novo synthesis of progesterone was demonstrated in our system by the significant incorporation in vitro of acetate-1-C\textsuperscript{14}, a point also established by Sweat et al. (6) while our work was in progress.

Addition to the incubation medium of three gonadotropin preparations was found to increase significantly progesterone synthesis in luteal tissue slices above that of control tissues to which none was added (Columns 3 to 6). It may be noted that, although the concentrations of gonadotropins used in the experiments described in Table I are high, we have achieved stimulation of biosynthesis at significantly lower levels of both luteinizing hormone and human chorionic gonadotropin. However, minimal effective concentrations have not yet been established. Addition of 5 units of porcine follicle-stimulating hormone (Armour Laboratories, Chicago, Illinois, lot no. 377-201) to the incubation medium, in a single experiment, also was found to stimulate synthesis. This may well be due to its luteinizing hormone component, reported to be 3 to 4% of the activity of an equivalent weight of Armour luteinizing hormone standard. On the other hand, this effect may be a true stimulation by the follicle-stimulating hormone component. The addition of adrenocorticotropic hormone (commercial ACTH, 1 unit per 5.0 ml of medium) or of bovine serum albumin (0.2 mg per 5.0 ml of medium) did not increase the steroid biosynthesis of bovine luteal tissue above that of controls. It is therefore concluded that progesterone synthesis by surviving luteal slices can be stimulated by the addition in vitro of both pituitary and urinary gonadotropin preparations, and that this demonstration reflects the similar action of the hypophysial hormones upon luteal tissue in the intact animal (7).

The role of certain cofactors in the action in vitro of ACTH on the biosynthesis of corticosteroids in adrenocortical tissue slices (8) prompted us to explore the effect of TPNH on the synthesis in vitro of progesterone by luteal tissue. As shown in Column 7 of Table I, a TPNH-generating system consisting of TPN, glucose-6-P, and a crude glucose-6-P dehydrogenase ("hexokinase type II") was added to the luteal tissue preparations. The specificity of TPNH was shown by the failure of DPN or DPNH, in concentrations comparable to those of added TPNH, to stimulate progesterone synthesis. We are unable to explain this disagreement with the observations of Duncan et al. (1), who have reported that DPN increases the rate of progesterone synthesis in now luteal tissue.

In some experiments, it was found that certain corpora lutea did not respond to added gonadotropin or showed very small increases in biosynthetic rates. These same tissues, however, invariably responded fully to the addition of TPNH. This interesting phenomenon may be related to the age of the corpus luteum and is under study at this time. The variability of response may explain the inability of Duncan et al. (9) to demonstrate a stimulation of progesterone synthesis by gonadotropin preparations.

**REFERENCES**

A Crystalline Flavin Pyruvate Oxidase*

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(Received for publication, April 4, 1961)

Previous communications from this laboratory have reported the recognition and partial purification of a flavoprotein pyruvate oxidase which catalyzes the oxidative decarboxylation of pyruvate to acetate and CO2 (1, 2). This enzyme is obtained from an acetate auxotroph of *Escherichia coli* and is formed in response to the accumulation of pyruvate in the growth medium (3). We now wish to report the isolation of this flavoprotein in crystalline form and to describe some of the properties of the purified enzyme.

The purified flavoprotein has the typical behavior of a globular protein. It is insoluble in aqueous solutions of low ionic strength but readily dissolved in solutions of ionic strength of 0.2 and higher. Purification and crystallization of the flavoprotein is accomplished by the following steps: (a) rupture of the *Escherichia coli* cells by grinding with glass beads in 0.02 M potassium phosphate buffer, pH 7, in a colloid mill (4); (b) preparation of a 0.25 to 0.75 saturated ammonium sulfate fraction; (c) protamine sulfate treatment to remove nucleic acids; (d) preparation of a 0.36 to 0.55 saturated ammonium sulfate fraction; (e) heat treatment at 60° for 5 minutes to denature inactive protein; (f) chromatography on DEAE-cellulose at pH 5.7 with a gradient elution between 0.02 m and 0.3 m potassium phosphate buffer; (g) protamine sulfate precipitation and elution of the flavoprotein with 0.2 m potassium phosphate buffer, pH 5.7; and (h) dilution and crystallization of the flavoprotein in 0.05 to 0.1 m potassium phosphate buffer. A typical purification procedure for preparation of the crystalline enzyme is given in Table I. A photograph of the crystalline protein showing the rhombohedral character of the crystals is shown in Fig. 1.

The crystalline flavoprotein has a molecular weight of approximately 265,000 as determined by the Archibald method (5). The sedimentation constant (s₂₀,₅₀) of the enzyme in 0.2 M potassium phosphate buffer, pH 5.7, is 11.3 S. Flavin analyses (6-8) performed on the enzyme indicate that FAD is the sole flavin component and is present in a ratio of 4 moles of FAD per mole of enzyme. The crystalline enzyme does not contain thiamine pyrophosphate but has an absolute thiamine pyrophosphate requirement for reduction of the enzyme-bound FAD.

The reduced flavoprotein-enzyme complex is nonoxidizable. However, in confirmation of previous reports (9, 10), it is air oxidizable in the presence of a cytochrome-containing particulate fraction or in the presence of a soluble cytochrome b₅ preparation derived from the particulate fraction. When fully saturated with soluble cytochrome b₅, the flavoprotein has a turnover number of 10,000 (moles of pyruvate oxidized per minute per mole of enzyme). The incubation of reduced flavoprotein with soluble cytochrome b₅ leads to the oxidation of the flavoprotein via a cytochrome pathway. These results suggest that oxidation of the flavoprotein in vivo is accomplished via a cytochrome pathway.

When supplemented with pyruvate as substrate and thiamine pyrophosphate as coenzyme, the purified flavoprotein will also react sluggishly with electron acceptors such as 2,6-dichlorophenolindophenol and ferricyanide (turnover number = 1800, ferricyanide). Maximal activity (turnover number = 53,000, ferricyanide) is achieved in these reactions by a variety of methods. Partial hydrolysis of the flavoprotein by trypsin (11) or chymotrypsin (12) produces an altered protein which is approximately 25 times more active than the native protein with ferricyanide as electron acceptor. Exposure of the flavoprotein to certain ionic surface active agents, such as sodium lauryl sulfate, also converts the flavoprotein to an activated form in the ferricyanide reaction. Finally, the cytochrome containing particulate fraction (10), soluble cytochrome b₅ (10), or lipid extracts derived from the particulate fraction (13) activate the flavoprotein in the ferricyanide reaction. The mechanism of activation of the flavoprotein by these various agents is under investigation.

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† Predoctoral Research Fellow of the National Institutes of Health, United States Public Health Service.


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**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
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<th>Protamine sulfate supernatant</th>
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<th>Eluate fraction from protamine sulfate</th>
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