Hydrolysis of the first cut of the low boiling ethereal material gave a pale yellow oil (86%) which, after trituration with hexane at −70°, formed colorless needles melting when warmed to room temperature.

Hydrolysis of the last cut of low boiling ethereal material (b.p. 94–97° at 0.3 mm.) produced colorless needles (71%) of γ-methylallyl 2-carboxy-6-methylphenyl ether, m.p. 74.5–75°, identical with an authentic sample by mixed m.p.

Hydrogenation of a sample of the high boiling ethereal fraction proceeded with the uptake of 3 mole equivalents of hydrogen. Hydrolysis of the hydrogenated product yielded VI, thereby identifying the high boiling ethereal material as diallylated substance(s) (*cf.* ref. 3).

Preferential Rearrangement.—A sample of the low boiling ethereal material from the reaction of α-methylallyl chloride and methyl *o*-cresotinate was subjected to a preferential rearrangement at 120° (see ref. 3 for procedure). After 27 hours a control on the pure γ-methylallyl ether indicated that it had suffered 10% rearrangement and showed a blue-green ferric chloride test while the ethereal mixture showed a deep royal blue test. The refractive index of the mixture changed from $n^{20}D$ 1.5145 to $n^{20}D$ 1.5184 during the 27-hour period while that of the control sample changed from $n^{20}D$ 1.5178 to 1.5195. The preferentially rearranged material was worked up in the usual manner and on distillation gave a 23% yield of **2-carbomethoxy-4-(α-methylallyl)-6-methylphenol (III)**, colorless oil, b.p. 97–103° at 0.9 mm., $n^{20}D$ 1.5196, ferric chloride test deep blue.

Hydrolysis of a sample of III gave 97% of a crude solid which after repeated recrystallization from hexane formed colorless needles of **2-carboxy-4-(α-methylallyl)-6-methylphenol (VII)**, m.p. 93–94.3°. Admixture with pure V gave a m.p. of marked depression. A mixed m.p. with the lower melting acid obtained from the phenolic fractions showed no depression (*vide supra*).

Anal. Calcd. for $C_{12}H_{14}O_3$: C, 69.9; H, 6.8. Found: C, 69.6; H, 6.7.

Microhydrogenation of VII over Adams catalyst proceeded with the uptake of 1 mole equivalent of hydrogen to form **2-carboxy-4-(2-butyl)-6-methylphenol** melting, after recrystallization and sublimation, at 94.4–95.1°. Admixed with authentic VII it melted at 89–90°.

Anal. Calcd. for $C_{12}H_{16}O_3$: C, 69.2; H, 7.8. Found: C, 69.4; H, 7.9.

Ozonolysis.—The apparatus and procedure used for ozonolysis were those previously described.[3] Results are shown in Table I.

TABLE I

Sample	H_2CO found as dimethone deriv., %	Terminal methylene cpd., %
Allyl ether of methyl *o*-cresotinate (model cpd. for ethers)	56 ± 5.0	100
γ-Methylallyl ether (II)	0	0
Fract. 1 of the low boiling ethereal (mixt. of I and II)	31.2	55.3 ± 5
2-Carbomethoxy-4-allyl-6-methylphenol (model cpd. for phenols)	55.2 ± 0.3	100
2-Carbomethoxy-4-(γ-methylallyl)-6-methylphenol (IV)	0	0
2-Carbomethoxy-4-(α-methylallyl)-6-methylphenol (III)	37.5	67.9 ± 0.5

Infrared Spectra.[9]—Infrared spectra were obtained in the 2–15μ region with a Perkin–Elmer spectrophotometer, model 12C. Characteristic peaks in the significant 9–11.5μ region are shown in Table II.

The strong absorption bands at 10.86μ in the ether mixture from the α-ether preparation and at 10.87μ in the preferentially rearranged material confirm the presence of the α-methylallyl compounds. The absence of detectable absorption at 10.36μ in the spectrum of III affirms the preferential nature of the rearrangement of the mixture of I and II and strongly suggests that the main course in the rearrangement of I is a non-inverting one.

TABLE II

II	10.33μ (str)
Fract. 1 of low boiling ethereal (mixt. of I and II	10.06–10.30μ (mod str); 10.86μ (str) (broad absorption)
IV	10.36μ (str)
III	10.08μ (mod str); 10.87μ (str)

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(9) For a discussion of the characteristic infrared absorption associated with terminal methylene groups and internal double bonds in these compounds see ref. 3. Complete spectra for the compounds reported in this paper are contained in the Ph.D. dissertation of R. L. Crecelius, obtainable through Interlibrary Loan from the University of Wyoming Library.

A Transhydrogenase and Reduced Triphosphopyridinenucleotide Involved in the Oxidation of Desoxycorticosterone to Corticosterone by Adrenal Tissue[1]

BY MAX L. SWEAT AND MARION D. LIPSCOMB

RECEIVED JULY 6, 1955

Since the observation of Hayano, Dorfman and Prins[2] that adrenal slices and homogenates were capable of converting desoxycorticosterone to a substance with glycogenic activity, several laboratories have studied steroid conversions in adrenal a-cellular preparations. McGinty, *et al.*,[3] demonstrated the conversion of 17-hydroxy-11-desoxycorticosterone to 17-hydroxycorticosterone in beef adrenal homogenates. Sweat[4] was able to effect this conversion in a mitochondrial fraction and on the basis of heat, rate and inhibitor studies concluded that the conversion was enzymic in nature. He further pointed out that the reaction would not proceed if the fumarate moiety of the Hayano–Dorfman buffer medium were replaced with phosphate buffer. Recently, Hayano and Dorfman[5] have observed that oxidized TPN restores the activity of washed or aged preparations of adrenal tissue. Various laboratories[6–9] have reported that metabolites other than fumarate enhance the oxidation of 11-desoxysteroids. In this report we wish to present evidence that one of the cofactors in the conversion of desoxycorticosterone to corticosterone is reduced triphosphopyridinenucleotide and that one of the enzymes involved in generating this cofactor in adrenal tissue is a transhydrogenase.

Mitochondria equivalent to 40 mg. of adrenal tissue were suspended in 1 ml. of an incubation medium containing 0.04 *M* sodium phosphate buffer (*p*H 7.4), 0.004 *M* $MgCl_2$, 40 μg. of desoxycorticosterone and one or a combination of the following cosubstrates and cofactors: 0.01 *M* fumarate, malate, citrate, *cis*-aconitate, isocitrate

(1) This investigation was supported by a research grant (A-331) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

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(7) H. Schmidt and H. Staudinger, *Biochem. Z.*, **325**, 148 (1953).

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or α-ketoglutarate; 2.5×10^{-4} M triphosphopyridinenucleotide (TPN); 2.5 to 10.0×10^{-4} M reduced diphosphopyridinenucleotide (DPNH); 2.5 to 10.0×10^{-4} M reduced TPN (TPNH); 2.5×10^{-4} M TPN + 2.5 to 10.0×10^{-4} M DPNH. In one experiment, 0.0045 M glucose 6-phosphate and glucose 6-phosphate dehydrogenase were employed with 2.5×10^{-4} M TPN. The mixtures were incubated with agitation in 10-ml. flasks at a temperature of 35° for one hour. The corticosterone synthesized in the preparations was extracted with chloroform and analyzed by fluorescence.[10]

In Fig. 1 are data which demonstrate that the rate of oxidation of desoxycorticosterone is enhanced in the presence of either TPNH or the glucose 6-phosphate–TPN system. Additional evidence for the requirement of TPNH has been obtained by a study of the rates at which the tricarboxylic acids, citrate, *cis*-aconitate and isocitrate in the presence of TPN enhance the rate of desoxycorticosterone oxidation. The data in Fig. 2 show that isocitrate is the most effective of the three acids. Isocitrate is a well known substrate of a TPNH generating system. The lag periods noted in the data for citrate and *cis*-aconitate are consistent with the concept that these two acids are converted into isocitrate. α-Ketoglutarate which follows isocitrate in the sequence of the Krebs cycle, exhibits very little activity.

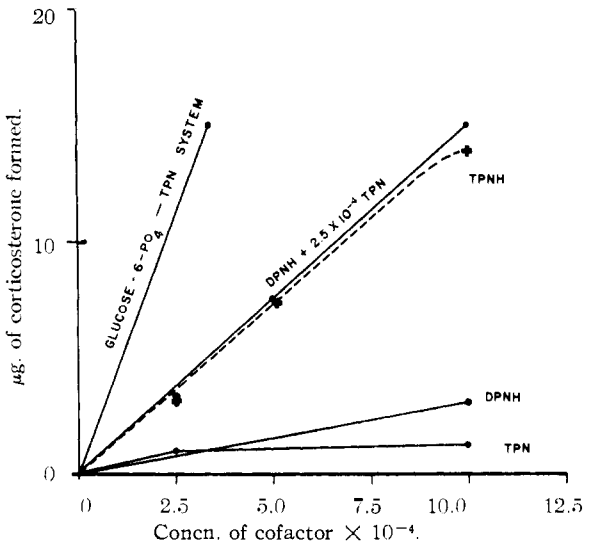

Fig. 1.—Oxidation of desoxycorticosterone in absence of "Krebs Cycle" acids.

In Fig. 2 are data which demonstrate fumarate and malate to be highly effective in enhancing the oxidation of desoxycorticosterone. It appears that these substances also exert their effect through their role in systems which generate TPNH. To explain the role of these metabolites, the following equations were considered

$$\text{Fumarate} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{Malate} \quad (1)$$

$$\text{Malate} + \text{DPN} \rightleftharpoons \text{oxalacetate} + \text{DPNH} \quad (2)$$

$$\text{DPNH} + \text{TPN} \rightleftharpoons \text{DPN} + \text{TPNH} \quad (3)$$

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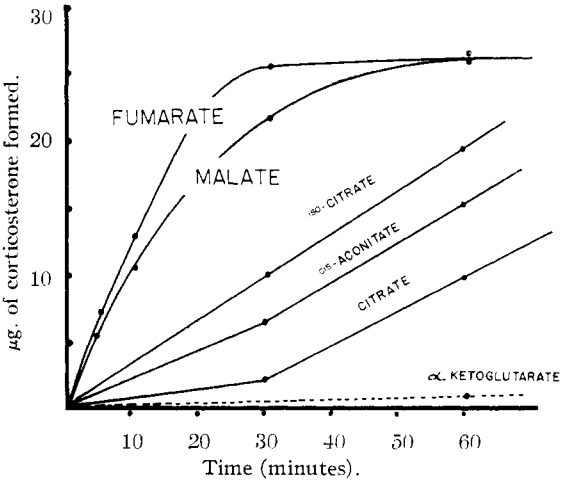

Fig. 2.—Relative rates at which co-substrates (0.01 M) enhance 11β-hydroxylation.

Reaction no. 3 requires the presence of a transhydrogenase enzyme.[11] That such a system is present in adrenal tissue is apparent in Fig. 1. When either TPN or DPNH is added to incubation mixtures without added fumarate or malate, only trace effects are observed. However, when trace quantities of TPN (0.5 to 2.5×10^{-4} M) are added to 2.5 to 10×10^{-3} M DPNH, a significant increase in activity is noted.

It appears from the above data that in the 11-β-oxidation of desoxycorticosterone the effects of the di- and tricarboxylic acids are largely due to their role in supplying reduced TPN. Consistent with this conclusion is the observation that potassium ferricyanate, methylene blue, toluidine blue, azure I, methyl viologen and benzyl viologen, all of which are effective electron acceptors, markedly inhibit the reaction.

The observation that fumarate and malate induce higher rates of activity than TPNH and isocitrate is not explained by the above data. These differences may be due to differences in enzyme levels or to permeability or other physical characteristics of the insoluble mitochondrial preparations. The differences in rate between the malate and fumarate systems may be due to the different rates at which oxalacetate (an inhibitor of 11-β-oxidation) is formed. Another possibility is that fumarate may act as a hydrogen acceptor in a later stage of the oxidation of the steroid.

The role of TPNH in the over-all oxidation of desoxycorticosterone remains unexplained.

The recent announcement of Hayano and Dorfman[12] that they have been successful in preparing a soluble enzyme system which does not require fumarate has been confirmed by us. However, in our opinion the slight activity observed in aqueous extracts (soluble?) of acetone powders without added fumarate represents the same small degree of activity which has been observed in mitochondrial preparations without added fumarate. Such activity is probably due to the presence of small

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quantities of key cofactors present in the preparations. It appears unlikely that soluble extracts are less dependent upon cofactor generating systems than the more intact preparations.

Acknowledgment.—The authors wish to express their thanks to Dr. Howard Gest for his valuable suggestions pertaining to this investigation.

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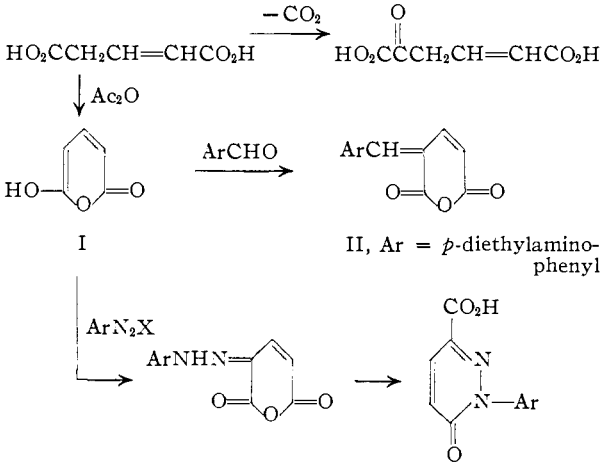
2-Pyrones. XVI. Benzylidene and Arylhydrazone Derivatives of Glutaconic Anhydride

BY RICHARD H. WILEY AND HENRY G. ELLERT

RECEIVED MAY 18, 1955

The procedures used previously[1,2] for the conversion of β-methylglutaconic anhydride to substituted benzylidene derivatives by condensation with aryl aldehydes and to arylhydrazone derivatives by coupling with aryl diazonium salts have been extended to glutaconic anhydride I. The preparation and characterization of the products obtained in these reactions and in the rearrangement of the arylhydrazones III to pyridazonecarboxylic acids IV are described in this report. The glutaconic anhydride used in these studies was prepared from glutaconic acid by anhydride interchange with acetic anhydride. The acid, which is available *via* several routes,[3] was prepared by hydrolysis and decarboxylation of diethyl oxalocrotonate[4] prepared in turn from ethyl oxalate and ethyl crotonate.[5]



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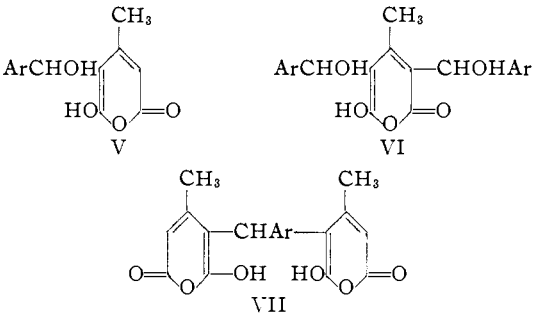
The reaction between glutaconic anhydride and *p*-diethylaminobenzaldehyde gave a purple product II, m.p. 219°, recrystallizable from toluene–petroleum ether. Although crystalline products were obtained from 3,4-dimethoxy- and *p*-dimethylaminobenzaldehydes, neither of these analyzed in acceptable agreement with the arylidene structure II. Apparently these products are too unstable to permit separation of analytically pure individual compounds from the mixtures formed by any techniques we have been able to devise to date. Varying analytical data were obtained on products obtained by altering minor details of the preparation.

In these reactions with aromatic aldehydes, the presence of the β-methyl group in the glutaconic anhydride clearly contributes to the ease with which characterizable arylidene derivatives are formed. This is probably partly a simple steric effect in which the β-methyl group shields the α-position from a continuing reaction. If, however, the products consist of mixtures of mono- and disubstituted products of the types V, VI and VII, a likely possibility corresponding to condensation of



aldehydes with *o*- and *p*-positions of phenols, then the β-methyl group, by virtue of its electron-releasing characteristics, may facilitate dehydration of V thus inhibiting formation of products such as VI, or dehydrated forms thereof, and VII. It is unlikely that any reaction with the aldehyde can occur in the free β-position.

By way of contrast the condensations of glutaconic anhydride with diazonium salts to give γ-phenylhydrazono structures III proceeds as does the reaction with β-methylglutaconic anhydride. Using similar procedures, yields of 57.3 to 87% were obtained. The products are formulated as phenylhydrazones on the basis of observations noted with the β-methyl types. Rearrangement of these products to the corresponding 1-aryl-2-pyridazone-5-carboxylic acids (IV) takes place in 28 to 74% yields.

Experimental[6]

γ-(4′-Diethylaminobenzylidene)-glutaconic Anhydride (II, Ar = *p*-Diethylaminophenyl).—A solution of 0.5 g. (0.00415 mole) of glutaconic anhydride and 0.73 g. (0.00415 mole) of *p*-diethylaminobenzaldehyde in 10 ml. of 95% ethanol immediately deposited a deep red precipitate. This precipitate was collected and recrystallized from toluene–petroleum ether to give 0.80 g., 66%, of γ-(4′-diethylaminobenzylidene)-glutaconic anhydride, m.p. 219°.

Anal. Calcd. for $C_{16}H_{17}O_3N$: C, 70.83; H, 6.32. Found: C, 70.57; H, 6.58.

γ-Ketoglutaconic Anhydride Phenylhydrazone (III, Ar = Phenyl).—A solution of 0.47 g. (0.005 mole) of aniline in 25

(6) Analyses by Micro Tech Laboratories, Skokie, Ill.