THE ASSAY OF ESTRADIOL-SENSITIVE TRANSHYDROGENASE

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AN ESTRADIOL-POTENTIATED transhydrogenase is found in placenta.[3] Gordon and Villee[2] have shown that the estradiol-potentiated reaction is characterized by the disappearance of tricarboxylic acid, the appearance of α-ketoglutaric acid (KG), an absolute requirement for diphosphopyridine nucleotide (DPN), and catalysis by certain divalent ions. They have used the placental enzymatic system for the estimation of estradiol in tissues and urinary extracts.[2]

In order to carry out quantitative studies of this enzyme in diseased tissues, the optimum conditions for assay were studied. Human placenta was utilized for these studies because it was found to be a constant and reliable source of enzyme.

METHODS AND MATERIALS

Isocitric and *cis*-aconitic acids were obtained from commercial sources and assayed enzymatically. Isocitrate was determined with isocitric dehydrogenase triphosphopyridine nucleotide (TPN). *Cis*-aconitate was determined with isocitric dehydrogenase and aconitase.[10] Pure *d*-isocitric acid lactone was obtained through the courtesy of Hubert Vickery and converted to the free acid by alkaline hydrolysis. Solutions of isocitrate and *cis*-aconitate were prepared on the bases of enzymatic assay and stored at −20° C. after adjustment of the *p*H to 6.8.

The source of enzyme was the supernatant fraction of placental tissue homogenate. Term placentas were rinsed of excess blood with 0.25*M* sucrose, and a 20% homogenate was prepared in this medium. Homogenization was carried out for 20 seconds in Potter type homogenizers with Teflon pestles. The homogenate was strained through gauze and centrifuged for 20 minutes at 31,000×*g*. The supernate was dialyzed for 15 hours against 2×50 volumes of 0.006*M* phosphate buffer, *p*H 7.0, in 0.25*M* sucrose. Following these operations in a 2° C. cold room, the supernate was stored at −20° C. Water for use in enzymatic assays was prepared from laboratory distilled water by redistillation in an all glass system.

DETERMINATION OF α-KETOGLUTARATE

The KG was determined by a modification of the method of Robins et al.[9] A stock solution was prepared by dissolving 34.8-mg.

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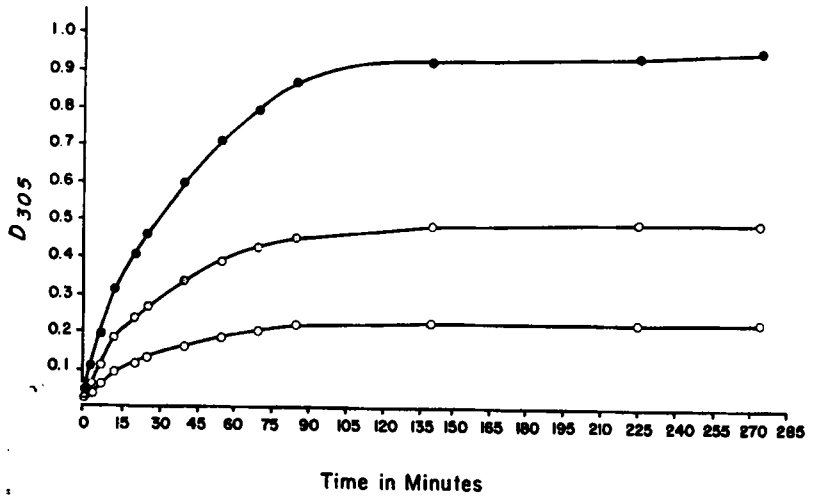

FIG. 1. Time course of reaction of 3-quinolinehydrozine hydrochloride with KG. Reagents added as in text. Open circles, 0.02 μ*M*; dotted circles, 0.04 μ*M*; solid circles, 0.08-μ*M* KG.

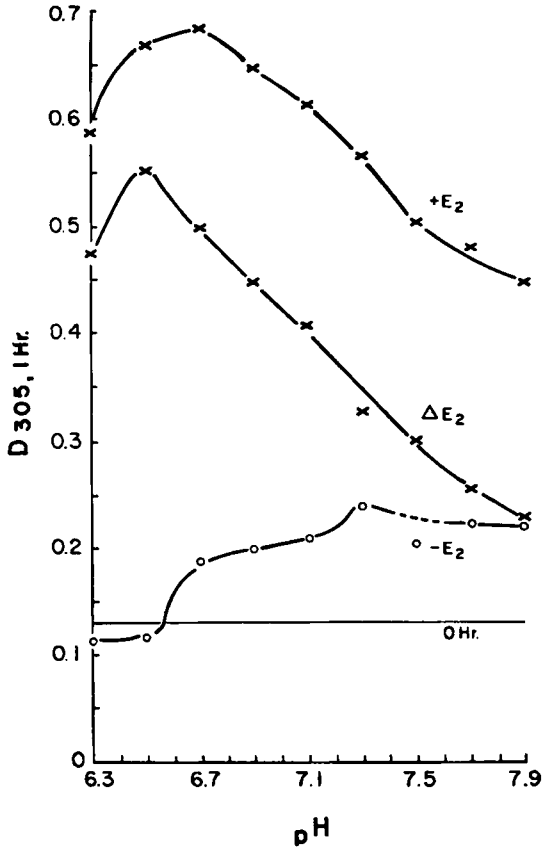

FIG. 2. The effect of *p*H on estradiol effect. The KG production is expressed as optical density at 305 mμ. An optical density of 1.0 is equivalent to 0.22-μ*M* KG. The assay system contained 6.6-μ*M* phosphate buffer at indicated *p*H, 1.3-μ*M* *d*-isocitrate, 0.24-μ*M* DPN, 4.2-μ*M* magnesium chloride, 23.8-μ*M* nicotinamide, 0.30-ml. 20% placental tissue homogenate, and estradiol, where indicated, at 1 μg. per ml. in a total volume of 1.0 ml. Incubation was for 1 hour at 30° C.

3-hydrazinoquinoline dihydrochloride (Eastman Kodak) in 5-ml. 0.1*N* hydrochloric acid. This solution was stored at −20° C. and made fresh every 10 days. A working solution was prepared daily by mixing 0.6 ml. of the stock solution with 9.0-ml. 0.1*N* hydrochloric acid and 4.5-ml. water. This solution should be very light yellow in color. Enzyme incubation mixtures were precipitated by the addition of 0.25 ml. of 20% metaphosphoric acid per ml. of the reaction mixture. The tubes were centrifuged for 10 minutes, and 0.4 ml. of deproteinized supernate was mixed with 0.1 ml. of the diluted 3-quinolinehydrazine hydrochloride. After 3 hours at room temperature, the samples were diluted with 0.4-ml. 0.01*N* hydrochloric acid, and the optical density was determined at 305 mμ against a reagent blank. This procedure permits 95% recovery of KG added to metaphosphoric acid–precipitated enzyme reaction mixtures and is sensitive to 0.01-μ*M* KG. Figure 1 shows the optical density (D_{305}) obtained with KG during color development. It has not been possible to speed up the reaction so that it is complete in less than 3 hours. Dilution with 0.01*N* hydrochloric acid at the end of 3 hours reduces the blank value materially. The procedure has been easily adapted to 0.001-μ*M* KG by reduction of the aforementioned volumes and the use of micro absorption cells.

TABLE 1

EFFECT OF ESTRADIOL ON PLACENTAL TISSUE HOMOGENATE DETERMINED BY DPNH AND KG PRODUCTION

Substrate	DPNH due to estradiol, μ*M*	KG due to estradiol, μ*M*
Citrate	0.021	0.025
Isocitrate	0.027	0.021
cis-Aconitate	0.027	0.025

RESULTS AND DISCUSSION

Comparison of DPNH *and* KG *Production.* In order to justify the use of KG production as an index of enzymatic activity, DPNH and KG production were compared. These should be equal if the reaction follows the course:

d-isocitrate+DPN→DPNH+KG

For this experiment, the extra DPNH formed in the presence of estradiol was determined spectrophotometrically by the procedure of Gordon and Villee.[3] The enzymatic assay system contained 1-μ*M* tricarboxylic acid, 3-μ*M* cobaltous chloride, 100-μ*M* tris buffer at *p*H 7.4, 300-μl. placental enzyme, and appropriate additions of DPN and estradiol in 1.0-ml. total

TABLE 2

EFFECT OF METAL ON *p*H OPTIMUM FOR α-KETOGLUTARATE PRODUCTION*

Molarity of buffer	Tris buffer		Phosphate buffer	
	*p*H		*p*H	
Catalyst	7.4	6.8	7.4	6.8
0.10*M*, Co^{++}	0.073	0.059	...	...
0.10*M*, Mg^{++}	0.081	0.069	0.053	0.073
0.005*M*, Co^{++}	0.110	0.092	...	...
0.005*M*, Mg^{++}	0.091	0.115	0.114	0.130

*The assay system consisted of 0.30-ml. dialyzed placental homogenate, metal at 5 μ*M* per ml., estradiol 2 μg. per ml., DPN 0.30 μ*M*, isocitrate 1 μ*M*, total volume 1.0 ml. The incubation time was 2 hours at 30° C. Co^{++} was not tested in phosphate buffer because of precipitation. The effect is expressed as micromoles KG produced in 2 hours as a result of estradiol.

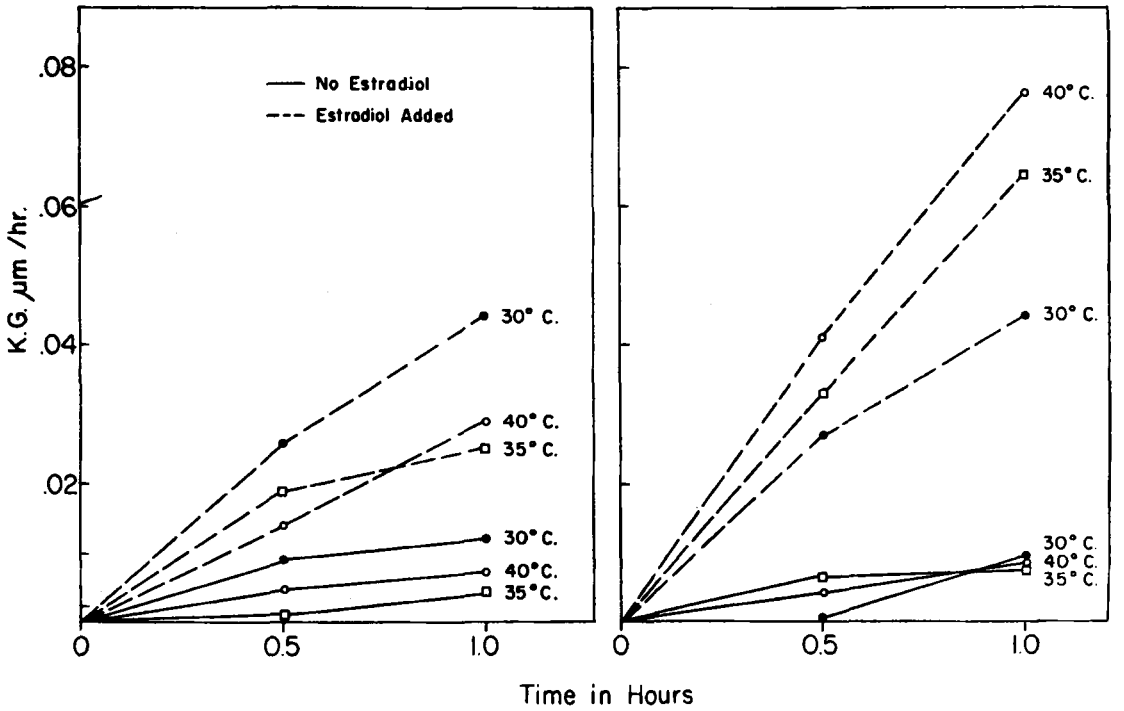

FIG. 3. The effect of temperature on estradiol stimulation of KG production. The figure to the left shows an assay on a fresh placental tissue homogenate centrifuged for 30 minutes at 54,000×*g*. The figure on the right shows the results on the same preparation after 5 days storage at −20° C. The assay system contained 6.6-µ*M* phosphate buffer at *p*H 6.8, 0.8-µ*M* isocitrate, 0.24-µ*M* DPN, 4.2-µ*M* magnesium chloride, and 0.30-ml. 20% placental tissue homogenate in a total volume of 1.0 ml. Incubation was for 1 hour.

volume. The first cuvette was employed for optical balance. It contained placental tissue homogenate, buffer, and cobaltous chloride (Co^{++}). The second cuvette contained, in addition, substrate and 0.30-µ*M* DPN. The third cuvette was identical with the second except that it contained 1.0-µg. estradiol. DPNH formation as a result of estradiol was determined by observing the difference in optical density between the second and third cuvettes at 340 mµ during 30 minutes of incubation at room temperature. At the end of the incubation period 0.25-ml. 20% metaphosphoric acid was added to each tube, and the KG was determined in the supernate. Table 1 shows that the reaction took the expected stoichiometric course.

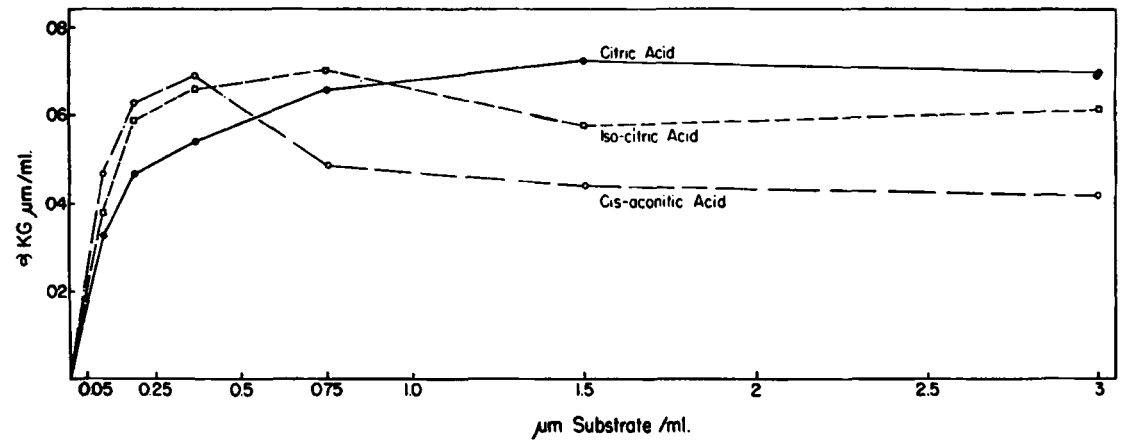

FIG. 4. The effect of substrate concentration on KG production. The enzymatic assay system consisted of 6.6-µ*M* phosphate buffer at *p*H 6.8, 0.30-µ*M* DPN, 4.2-µ*M* magnesium chloride, and 0.30-ml. 20% placental tissue homogenate. The substrate, adjusted to *p*H 6.8, was added as indicated, for a total volume of 1.0 ml. Incubation was for 1 hour at 30° C. The result is expressed as micromoles KG produced as a result of 1 µg. per ml. estradiol.

TABLE 3

THE EFFECT OF CERTAIN SUBSTANCES ON PLACENTAL ISOCITRIC DEHYDROGENASE

Substance	Concentration	Inhibition of estradiol effect, %
Nicotinamide	$2\times10^{-2}M$	0
Arsenite	3×10^{-3}	0
Versene	1×10^{-2}	50-100
Versene	1×10^{-3}	0
Vitamin D_2	2.5×10^{-4}	0
Propylene glycol	$1\times10^{-1}M$	0

Effect of pH. Figure 2 shows the effect of *p*H on KG production in phosphate buffer, catalyzed by magnesium chloride (Mg^{++}). Increase of *p*H in the interval 6.5 to 7.3 caused increased KG production in the vessels from which estradiol was omitted. The vessels that contained estradiol showed a maximum production of α-ketoglutarate at 6.7. Optimal estradiol effects were consistently seen in the region of 6.5 to 6.8. A similar sharp *p*H requirement, around *p*H 7.4, was observed by Gordon and Villee[3] using tris buffer, cobaltous chloride catalysis, and optical measurement of DPNH production. Table 2 shows that optimum *p*H for the estradiol effect varies with the buffer, as well as with the nature and concentration of the metal activator.

Effect of Incubation Temperature. Several experiments were done to determine the optimum temperature for the assay of estradiol sensitivity. The results are not consistent, since different enzyme preparations showed markedly different behavior with variation of temperature. The uncatalyzed production of α-ketoglutarate is insensitive to temperature while the estradiol-catalyzed reaction varied with the preparation assayed. Figure 3 shows the results obtained with a fresh placental supernate contrasted with the same preparation after 5 days storage at −20° C. In the fresh preparation, the maximum rate of KG production occurred at 30° C., while after storage the same homogenate demonstrated an apparent doubling of the rate between 30° and 40° C. Tissue homogenates that have been centrifuged at 29,000×*g* have shown better thermal stability than preparations centrifuged at 54,000×*g*. The latter often show optimum KG production at 30° C. rather than at 40° C. The tissue homogenates are obviously complex enzyme systems with variable degrees of thermal stability. The practical temperature for assay is 30° C.

Substrate Concentration. Figure 4 shows an example of the effect of tricarboxylic acid concentration on the rate of uncatalyzed ketoglutarate production. The maximum rate of estradiol stimulation is achieved with *cis*-aconitate and isocitrate at levels between 0.4 and 0.8 μ*M* per ml. with different homogenates. The maximum rate of estradiol stimulation is achieved at 1.5 to 2.0 μ*M* per ml. of citrate. The utilization of citrate and *cis*-aconitic acids by the estradiol-sensitive isocitric dehydrogenase presumably requires the action of aconitase prior to the action of the hormone-sensitive enzyme. For this reason, isocit-

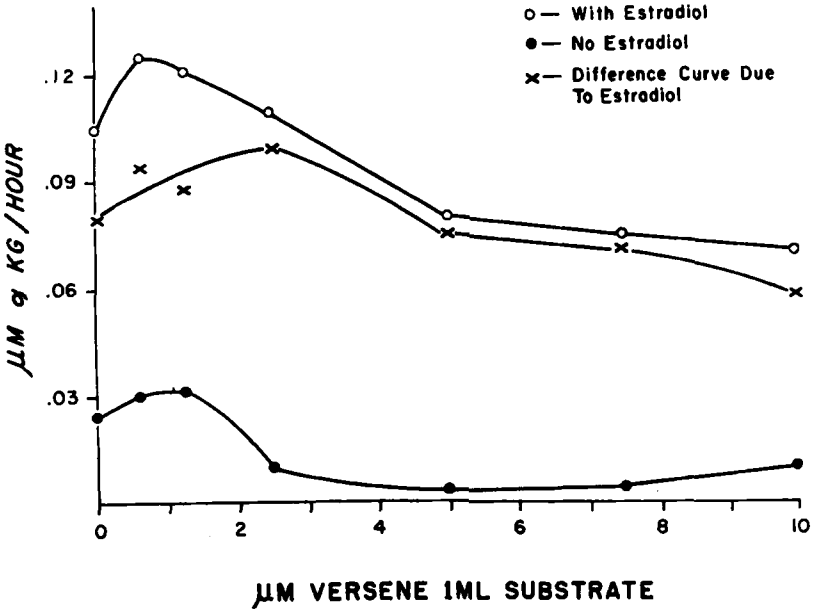

FIG. 5. The effect of versene concentration on estradiol effect. The assay system consisted of 7-μ*M* phosphate buffer at *p*H 6.8, 0.30-μ*M* DPN, 7-μ*M* magnesium chloride, 0.30-ml. 20% placental tissue homogenate, 0.7-μ*M* *d*-isocitrate, and estradiol, where indicated, at a level of 5 μg. per ml. Incubation was for 1 hour.

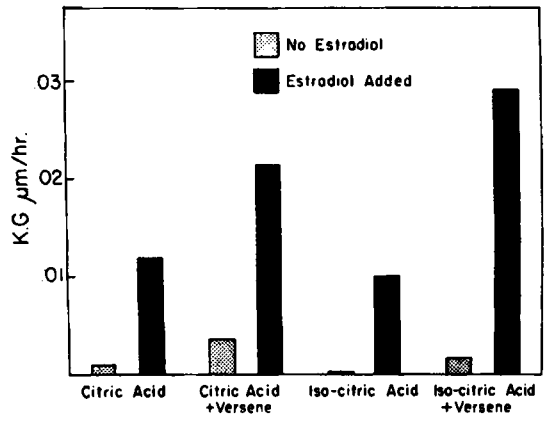

FIG. 6. The effect of versene on estradiol effect under the same conditions as in Fig. 4. Citrate and isocitrate at 0.7 µ*M* per ml.

rate at 0.7 µ*M* per ml. of the enzymatically active dextrorotatory form is employed for assay. The enzymatic assay of commercial isocitrate prior to its use in this analysis is important since many of these preparations are impure. Pure *d*-isocitrate gives the same result as *dl*-isocitrate when the concentration of the latter is expressed in terms of enzymatic assay that measures only the *d*-isomer.

Estradiol Concentration. Our results confirm those of Gordon and Villee[3] in that 1 µg. per ml. estradiol gives a maximum stimulation of α-ketoglutarate production. Estradiol is added to substrate solutions from a stock solution in propylene glycol. The latter should not exceed 1% by volume of the final reaction mixture.

Cofactors and Inhibitors. Coenzyme A, adenosine monophosphate, adenosine triphosphate, thiamine, pyridoxine, thioctic acid, chorionic gonadotropin (Ayerst), and flavine adenine dinucleotide were tested at *p*H 6.8, with 0.007*M* phosphate buffer, 0.001*M* isocitrate, 0.006*M* magnesium chloride, and estradiol, 1 µg. per ml., with placental tissue homogenate representing 70 mg. of tissue per ml. When the cofactors were added at 170 µg. per ml. incubation for 1 hour at 30° C. showed no change in the estradiol effect. No cofactors have been found other than the requirement for DPN and divalent ions. Talalay and Williams-Ashman[11] have shown recently that the mechanism of the estradiol sensitivity involves the transfer of hydrogen from a catalytic amount of reduced triphosphopyridine nucleotide (TPNH) to DPN. Isocitric dehydrogenase TPN acts as a TPNH-generating system. In the studies reported here the dialyzed placental enzyme contained an adequate amount of TPN in order for the transhydrogenase mechanism to operate. Further addition of TPN did not enhance activity in these preparations.

The effect of several other substances that might be of importance in the practical measurement of this enzyme is illustrated in Table 3. Nicotinamide was added to the assay system for the study of tumor homogenates containing diphosphopyridine nucleotidase (DPNase).[7] At a level of 0.02*M* nicotinamide there was no significant inhibition of estradiol effect. Arsenite ($3\times10^{-3}M$) inhibited the conversion of ketoglutaric acid to succinate in homogenates.[8] In all centrifuged homogenates studied so far, α-ketoglutarate utilization has not been a problem, but the effect of arsenite was studied for use with crude homogenates. At this concentration of arsenite there was no inhibition of the estradiol effect.

Versene (ethylenediaminetetraacetic acid) was studied in an effort to find an effective substance for the removal of inhibitory metals from homogenates. Figure 5 shows considerable inhibition of estradiol effect at $10^{-2}M$ versene but little effect at concentrations below $2.5\times10^{-3}M$. Some placental homogenates were unaffected by 0.001*M* versene but Fig. 6 shows a typical preparation that showed considerable enhancement of estradiol sensitivity at this concentration of versene. The result was probably due to the presence of some metal inhibitor. These data justify addition of 0.001*M* versene to the assay system.

De Luca and Steinbock[1] have demonstrated decreased citrate and isocitrate utilization and decreased KG production by kidney mitochondria in the presence of vitamin D_2. This substance, at a level of 100 µg. per ml. did not

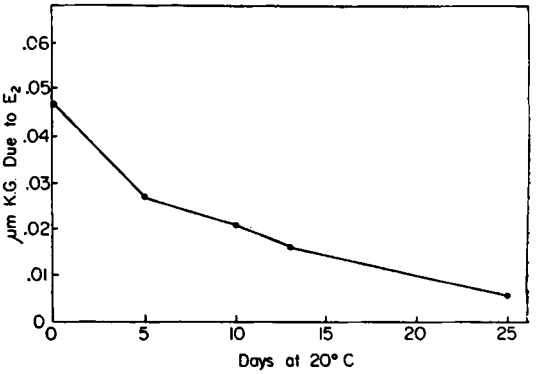

FIG. 7. The result of storage at 20° C. on estradiol effect under the same conditions as in Fig. 5.

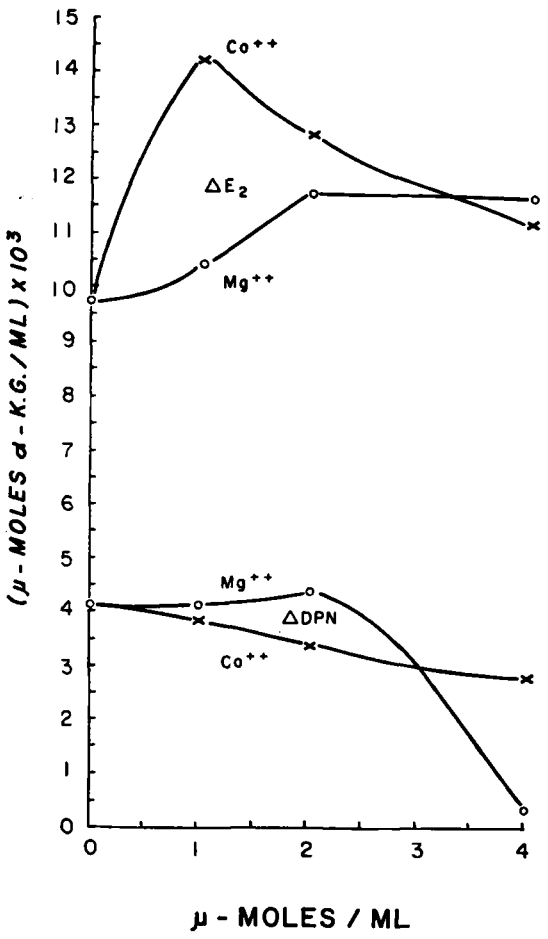

FIG. 8. The upper curves represent the estradiol effect with varying concentrations of cobaltous chloride (Co^{++}) and magnesium chloride (Mg^{++}). The lower curves represent the course of the reaction in the absence of estradiol. The assay system contained 7-μM phosphate buffer at *p*H 6.8, 0.7-μM *d*-isocitrate, 0.30-μM DPN, and 0.3-ml. placental tissue homogenate in a final volume of 1.0 ml. Estradiol, when present, was added at a level of 5 μg. per ml. The metal concentration is indicated in the figure.

inhibit the estradiol effect on isocitrate utilization in placental homogenate.

Propylene glycol had no effect at a final concentration of 0.1*M*. It is useful as a steroid solvent for addition to the enzymatic system.

Stability. Figure 7 shows the stability of the estradiol effect in a placental tissue homogenate stored at −20° C. Since there is gradual loss of activity under these conditions, homogenates are assayed immediately whenever possible or are stored at −20° C. for a maximum of 24 hours. Most samples of placenta showed little loss in enzymatic activity after storage at −20° C. for 24 hours.

Effect of Buffer and Ionic Strength. Tris (hydroxymethyl aminomethane) or phosphate buffer may be used to study the estradiol-sensitive enzymatic system. Table 4 shows that similar results are obtained with either buffer but that less estradiol effect is observed with 0.1*M* buffer than with 0.007*M* buffer. The addition of sufficient sodium chloride to bring the dilute buffer to the same ionic strength as the more concentrated phosphate buffer produced little change in the phosphate assay system but significant inhibition with the tris. These studies were done with 0.3 ml. of the indicated buffer at *p*H 6.6, 0.3-ml. dialyzed placental enzyme, a final molarity of 1 μM per ml. of isocitrate, 4 μM per ml. of magnesium chloride (Mg^{++}), 0.25 μM per ml. DPN, and a final volume of 1.0 ml. Estradiol was added to appropriate tubes at a level of 1 μg. per ml. Incubation was for 1 hour at 30° C. Although tris does not operate as an efficient buffer at *p*H 6.6, the actual *p*H change during reaction was less than 0.1 *p*H unit in either assay system.

Effect of Metals. The estradiol stimulation of ketoglutarate production is quite sensitive to metal concentration. Figure 8 shows the effect of cobaltous chloride (Co^{++}) or magnesium chloride (Mg^{++}) concentration on the rate of α-ketoglutarate production by the hormone-catalyzed assay system. The optimum Co^{++} concentration varied in different placental tissue extracts between 1 and 5 μM per ml. As much as 7 μM per ml. of Mg^{++} were required for maximum activity with some placental extracts. Increase of Mg^{++} from 7 μM per ml. to 15 μM per ml. produced no decrease in the optimum production of KG. The lack of a maximum in the Mg^{++} curve makes this ion more useful than Co^{++} for enzymatic assay, although Co^{++} is a more effective catalyst under carefully defined optimum conditions. The effect of 11 metals on KG production by placenta is compared in Table 5. The metals were added to a final concentration of 4 μM per ml. In the case of iron (Fe^{++} and

TABLE 4

EFFECT OF BUFFER AND IONIC STRENGTH ON ESTRADIOL EFFECT

	Buffer					
	Phosp.	Sod. chl. +phosp.	Phosp.	Tris	Sod. chl. +tris	Tris
Final molarity of buffer	0.0066	0.0066	0.100	0.0066	0.0066	0.100
Total ionic strength	0.036	0.210	0.210	0.0048	0.210	0.082
Micromoles KG due to estradiol	0.052	0.054	0.031	0.058	0.028	0.008

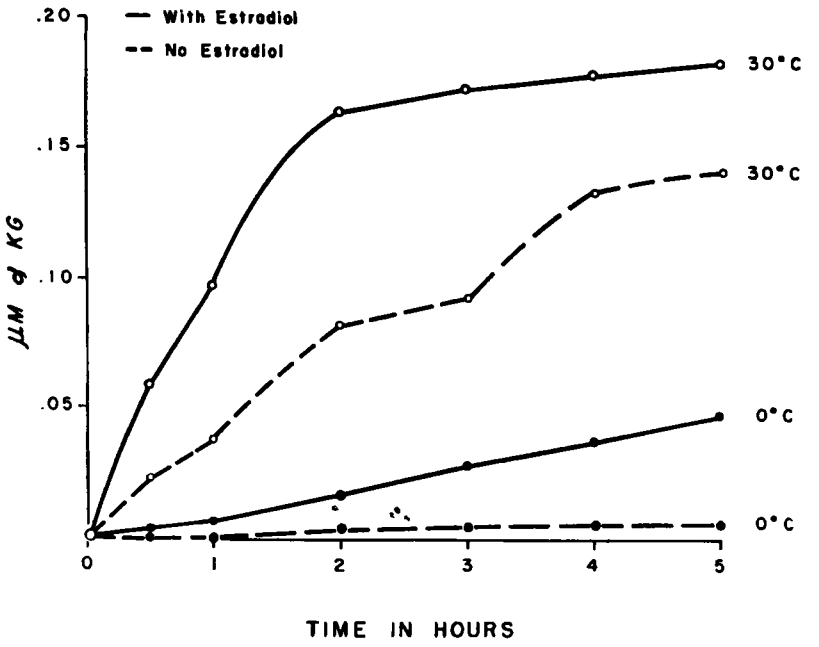

FIG. 9. The effect of incubation time on the estradiol effect under the same conditions as in Fig. 4.

Fe+++) some precipitate occurred with the buffer. The solutions of salts were adjusted to *p*H 6.8 before addition to the test system of *p*H 6.8 phosphate buffer, 7 μ*M* per ml.; isocitrate, 1 μ*M* per ml.; and DPN, 0.25 μ*M* per ml., in a total volume of 1.0 ml. Incubation was for 1 hour at 30° C. Estradiol, when present, was added at a level of 1 μg. per ml.

These data show that Co++, Mg++, Mn++, and Fe+++ have a catalytic function in the estradiol potentiation of isocitrate oxidation. The result with Fe+++ is not certain because it involved a large correction for color developed when Fe+++ was mixed with 3-quinolyl hydrazine. The inhibitory action of copper (Cu+++) has also been observed by Hagerman and Villee.[4]

Time of Incubation. Figure 9 shows the result when the estradiol stimulation of KG production is studied over a period of hours. In this experiment the reaction proceeded at a constant rate for 2 hours at 30° C. and then markedly decreased. Occasionally an enzyme preparation will continue at the initial rate for 4 hours, but assays of tissues are not carried out for longer than 2 hours. The reaction at 0° C. maintained a constant rate for 5 hours.

Concentration of Enzyme. Figure 10 demonstrates that the estradiol stimulation of α-ketoglutarate production is proportional to the enzyme concentration up to 20% homogenate. This experiment was done with Co++ added as the necessary divalent ion. Similar results have been obtained with Mg++. Because the reaction of interest is slow, it is necessary to use concentrated tissue homogenates. Assays should be done with 2 levels of tissue homogenate concentration to insure that the reaction mixture is operating in the linear range of α-ketoglutarate production.

Final Procedure. The basic reagent is prepared as indicated in Table 6. The final solutions are stored at −20° C. in small polyethylene bottles and are stable for at least 3 months under these conditions.

TABLE 5

THE EFFECT OF CATIONS ON PLACENTAL ISOCITRIC DEHYDROGENASE

Salt	KG produced	
	In absence of estradiol, $\mu M \times 10^{-3}$	In presence of estradiol, $\mu M \times 10^{-3}$*
None	6.1	0.0
Co^{++}	10.0	25.7
Mg^{++}	12.7	18.5
Mn^{++}	10.4	14.7
Fe^{+++}	9.6	14.4
Fe^{++}	8.5	5.0
Ca^{++}	9.6	−2.6
Cu^{++}	0.6	0.0
Zn^{++}	0.0	2.0
Ce^{++++}	19.6	−6.8
Al^{+++}	8.0	−1.0
Hg^{+++}	6.1	0.6

*Extra KG produced in excess of the amount in column 2.

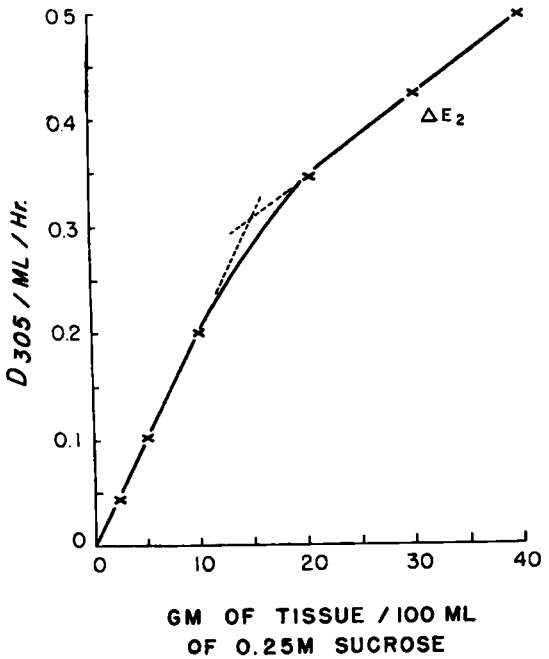

FIG. 10. The effect of tissue homogenate concentration on KG production. Results are expressed as changes in optical density at 305 mμ (D_{305}) for extra KG color resulting from estradiol. The assay system contained 7-μ*M* phosphate buffer at *p*H 6.8, 0.7-μ*M* *d*-isocitrate, 2-μ*M* cobaltous chloride, 0.30-μ*M* DPN, and estradiol, 5 μg. per ml., for a total volume of 1.0 ml.

The reagent without estradiol contained 1% by volume of propylene glycol. The reagent with estradiol contained the same amount of propylene glycol and a final concentration of 7.5-μg. estradiol per ml. At the time of the assay, the tubes of reagent were thawed, and 0.30-ml. substrate was pipetted into small test tubes in cracked ice. Tissue homogenate, 0.15 ml., was added at 2 appropriate concentration levels to each of the 2 reagents. In addition, blanks were prepared by adding homogenate to tubes containing 0.30-ml. substrate and 0.10-ml. 20% metaphosphoric acid. The reaction mixtures were incubated for an appropriate time and stopped with 0.10-ml. 20% metaphosphoric acid. The tubes were centrifuged and KG analysis was performed on the supernatant. Duplicate incubations checked within 10%.

TABLE 6

BASIC REAGENT USED FOR THE ASSAY OF THE EFFECT OF ESTRADIOL

Component	Amount, *M*
Sodium isocitrate	0.0010
Magnesium chloride	0.010
Phosphate buffer, *p*H 6.8	0.010
Sodium versenate, adjusted to *p*H 6.8	0.0015
Nicotinamide	0.03
DPN	0.00045

SUMMARY

The assay of placental tissue homogenate for estradiol-sensitive transhydrogenase has been studied. Optimum *p*H, buffer, temperature, and metal concentration have been established. Versene potentiated the estradiol sensitivity of some homogenates. A stable system that can be used to assay homogenates for estradiol sensitivity is described. α-Ketoglutaric acid (KG) production was equal to reduced diphosphopyridine nucleotide (DPNH) formation when tricarboxylic acid was consumed. α-Ketoglutaric acid was determined by a simple colorimetric procedure.

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