DEMONSTRATION OF A DPNH: α-KETOGLUTARATE OXIDOREDUCTASE ACTIVITY IN EMBRYONIC CHICK LIVER SUPERNATANT FRACTION*

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IN HIS STUDY OF THE DISTRIBUTION OF ENZYMES IN THE SUBCELLULAR FRACTIONS OF EMBRYONIC CHICK LIVER, SOLOMON (1959) OBSERVED GLUTAMIC DEHYDROGENASE (GDH) ACTIVITY WITH A pH OPTIMUM AT 7.6 IN MITOCHONDRIA. IN ADDITION, GDH ACTIVITY WITH AN ACID pH OPTIMUM WAS REPORTED IN THE SUCROSE SUPERNATANT FRACTION. THE RECOGNITION OF TWO GDH ISOZYMES IN MAMMALIAN LIVER MITOCHONDRIA, ONE EXTRACTABLE WITH WATER AND THE OTHER WITH DIGITONIN (HIRSCHBERG ET AL., 1964), PROMPTED A RE-EXAMINATION OF THIS ENZYME IN THE DEVELOPING CHICK LIVER. TWO MITOCHONDRIAL ISOZYMES WERE OBSERVED, BOTH WITH A pH OPTIMUM OF 7.5 - 8.2 (SHEID AND HIRSCHBERG, 1965).

IN THE SUCROSE SUPERNATANT FRACTION, ASSAYS FOR GDH DEMONSTRATED ACTIVITY WITH A pH OPTIMUM OF 5.2. THIS FRACTION ALSO CONTAINED GLUTAMIC-PYRUVIC TRANSAMINASE (GPT) AND GLUTAMIC-OXALOACETIC TRANSAMINASE (GOT) MEASURABLE AT THIS pH. THE SPECIFIC ACTIVITIES OF ALL THREE ENZYMES AT pH 5.2 WERE COMPARABLE TO EACH OTHER AT VARIOUS POINTS IN THE DEVELOPMENT OF THE CHICK EMBRYO. THE STANDARD SPECTROPHOTOMETRIC METHODS USED FOR ALL THREE ENZYME DETERMINATIONS EMPLOYED α-KETOGLUTARATE AS A SUBSTRATE TO INITIATE THE REACTIONS AND MEASURED THE OXIDATION OF DPNH. IN VIEW OF THE SIMILARITY OF THE ASSAY CONDITIONS AND RESULTS, AND THE FACT THAT GDH IS GENERALLY CONSIDERED A MITOCHONDRIAL COMPONENT (DE DUVE ET AL., 1962) MOST ACTIVE AT pH

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VALUES ABOVE NEUTRALITY, THE NATURE OF THIS ENZYME ACTIVITY APPEARED WORTHY OF FURTHER INVESTIGATION.

IN THIS COMMUNICATION, EVIDENCE IS PRESENTED AGAINST THE PARTICIPATION OF THE DEHYDROGENATION OR TRANSAMINATION PATHWAYS FROM α-KETOGLUTARATE TO GLUTAMATE IN THE CHICK LIVER SUPERNATANT FRACTION AT PH 5.2 BUT, INSTEAD, FOR THE DIRECT ENZYMATIC CONVERSION OF THE SUBSTRATE TO α-HYDROXYGLUTARATE IN THE PRESENCE OF DPNH.

METHODS

SPECTROPHOTOMETRIC MEASUREMENTS: SUBCELLULAR FRACTIONS WERE PREPARED FROM POOLED EMBRYONIC CHICK LIVER AT VARIOUS STAGES DURING DEVELOPMENT IN 0.25M SUCROSE (1:10) BY DIFFERENTIAL CENTRIFUGATION (SCHNEIDER AND HOGEBOOM, 1950). A WATER EXTRACT (MW) AND A DIGITONIN EXTRACT (MD) WERE OBTAINED FROM THE MITOCHONDRIAL PELLET (SHEID AND HIRSCHBERG, 1965).

THE GDH ASSAY MIXTURE (OLSON AND ANFINSEN, 1952) CONSISTED OF DPNH, NH_4^+, ENZYME, AND ACETATE OR PHOSPHATE BUFFER. AFTER 10 MIN. INCUBATION, α-KETOGLUTARATE, ADJUSTED TO THE PROPER PH, WAS ADDED WITH MIXING. THE OXIDATION OF DPNH WAS THEN FOLLOWED SPECTROPHOTOMETRICALLY AT 340 Mμ FOR THE FIRST FEW MINUTES. GOT AND GPT WERE ASSAYED SPECTROPHOTOMETRICALLY BY THE METHODS OF KARMEN (1955) AND WROBLEWSKI AND LADUE (1956), RESPECTIVELY. GOT WAS ALSO ESTIMATED COLORIMETRICALLY ACCORDING TO SHEID ET AL. (1965) AND GPT ACCORDING TO CALDWELL AND MCHENRY (1953). THE LINEARITY OF THE ENZYME ASSAYS WAS ESTABLISHED BY VARYING THE AMOUNTS OF ENZYME EXTRACTS IN INITIAL EXPERIMENTS.

CHROMATOGRAPHIC DETERMINATIONS: EMBRYONIC CHICK LIVER WAS HOMOGENIZED IN 0.1M ACETATE BUFFER, PH 5.2. THE SUPERNATANT FRACTION OBTAINED BY CENTRIFUGATION AT 105,000 X G WAS HEATED FOR 5 MINUTES AT 65°C, COOLED IN ICE, AND CENTRIFUGED BRIEFLY TO REMOVE COAGULATED PROTEIN. SPECTROPHOTOMETRIC ASSAYS SHOWED THAT THE RESULTING SUPERNATANT FRACTION CATALYZED THE OXIDATION OF DPNH AT PH 5.2 IN THE PRESENCE OF α-KETOGLUTARATE IN THE SAME MANNER AS SUCROSE SUPERNATANT FRACTIONS. THE ENZYME EXTRACT WAS DILUTED

1:10 and 0.2 ml was incubated with 90 μM α-ketoglutarate, 100 μM acetate buffer, pH 5.2, and 9 μM DPNH for 6 hours in a final volume of 3 ml. at room

TABLE 1. The effect of ammonium ion on true and artifactual glutamic dehydrogenase activity in the mitochondrial (water extract (Mw) and digitonin extract (Md)) and supernatant fractions of 19-day embryonic chick liver.*

Fraction	pH of Assay Mixture	NH_4^+ Addition**	Specific Activity (Average)†
Mitochondria (Mw)	5.2	+	0
Mitochondria (Mw)	5.2	-	0
Mitochondria (Mw)	7.6	+	540
Mitochondria (Mw)	7.6	-	0
Mitochondria (Md)	5.2	+	0
Mitochondria (Md)	5.2	-	0
Mitochondria (Md)	7.6	+	2950
Mitochondria (Md)	7.6	-	0
Supernatant	5.2	+	300
Supernatant	5.2	-	320
Supernatant	7.6	+	0
Supernatant	7.6	-	0

* Analogous results were obtained in duplicate assays of 14-day embryonic chick liver and of chick liver one day after hatching.

** The final concentration of NH_4^+, where added, was 0.15M.

† Specific Activity: E x 10^3/min/mg nitrogen. The results are an average of two duplicate determinations performed on separate days.

temperature. At this time, spectrophotometric assay indicated essentially complete disappearance of the added coenzyme. The reaction was stopped by the addition of 50% trichloracetic acid, the mixture centrifuged, and a sample of the supernatant fraction applied to Whatman No. 1 filter paper or Eastman activated Silica Gel Sheet Paper, Type K301R. Butanol-acetic acid-water (12:3:5) or ethanol-ammonia-water (16:1:3) were employed as solvents in ascending chromatography. Spots were identified with I_2 vapor, followed by bromcresol green or p-dimethylaminobenzaldehyde.

RESULTS AND DISCUSSION

TABLE 1 SHOWS THAT THE SUPERNATANT FRACTION REQUIRED NO NH_4^+ FOR THE OBSERVED ACTIVITY IN THE GDH ASSAY AT pH 5.2. IN CONTRAST, BOTH MITOCHONDRIAL FRACTIONS WERE DEPENDENT UPON THE ADDITION OF NH_4^+. AN ASSAY WITH THE CONWAY DIFFUSION TECHNIQUE ESTABLISHED THAT THE AMOUNTS OF NH_3 IN THE SUPERNATANT OR MITOCHONDRIAL FRACTIONS AT pH 5.2 OR 7.6 WERE NEGLIGIBLE, I.E. LESS THAN 0.6 γ/ML.

TABLE 2 ILLUSTRATES THE EFFECT OF KNOWN INHIBITORS AND ACTIVATORS OF GDH (TOMKINS AND YIELDING, 1961) ON THE ACTIVITY IN THE MITOCHONDRIAL AND SUPERNATANT FRACTIONS. DIETHYLSTILBESTROL (DES) HAD NO EFFECT ON THE SUPERNATANT ACTIVITY EVEN WHEN PREINCUBATED WITH ENZYME AT 37°C (TOMKINS AND YIELDING, 1961), BUT INHIBITED MITOCHONDRIAL ACTIVITY. ADENOSINEDIPHOSPHATE (ADP) STIMULATED GDH ACTIVITY IN BOTH MITOCHONDRIAL FRACTIONS, BUT NOT IN THE SUPERNATANT FRACTION. ETHYLENEDIAMINETETRAACETATE (EDTA) HAD A STIMULATORY EFFECT ONLY ON THE DIGITONIN MITOCHONDRIAL EXTRACT.

SOLOMON (1959) REPORTED LOW GDH ACTIVITY, ABOUT ONE-EIGHTH OF THE ACTIVITY FOUND AT pH 4.5-4.8, IN THE SUPERNATANT FRACTION AT pH 7.6. THIS ACTIVITY WAS CONFIRMED IN THE PRESENT EXPERIMENTS UNDER SOLOMON'S (1957) ASSAY CONDITIONS BUT WAS NOT MEASURABLE WITH THE OLSON-ANFINSEN (1952) METHOD.

ENZYME ACTIVITY MEASURABLE AT pH 5.2 DID NOT APPEAR IN THE MITOCHONDRIAL, RIBOSOMAL OR NUCLEAR FRACTIONS OF EMBRYONIC CHICK LIVER. IT WAS FOUND IN THE SUPERNATANT FRACTION OF EMBRYONIC DUCK LIVER, BUT NOT OF EMBRYONIC MOUSE OR RAT LIVER, ADULT CHICK, RAT OR MOUSE LIVER, AND THE MORRIS 7795 AND NOVIKOFF RAT HEPATOMAS.

GOT WAS READILY DEMONSTRABLE IN THE MITOCHONDRIAL AND SUPERNATANT FRACTIONS AT pH 7.6 BY EITHER THE SPECTROPHOTOMETRIC OR COLORIMETRIC METHODS; BUT ONLY THE FORMER, BASED ON A COUPLING REACTION INVOLVING OXIDATION OF DPNH, INDICATED MEASURABLE GOT ACTIVITY AT pH 5.2. FURTHER, ONLY THE SPECTROPHOTOMETRIC ASSAY OF GPT INDICATED ACTIVITY AT pH 5.2. THE ADDITION OF ISONIAZID, AN ANTAGONIST OF PYRIDOXAL PHOSPHATE (BRAUNSTEIN, 1960) AT A FINAL

CONCENTRATION OF 1 x 10^{-2}M, RESULTED IN 95% INHIBITION OF GOT ACTIVITY IN THE SUPERNATANT FRACTION AT pH 7.6, BUT HAD NO EFFECT ON THE ACTIVITY AT pH 5.2.

ADDITIONAL EVIDENCE AGAINST THE INVOLVEMENT OF GDH OR THE TRANSAMINASES IS PRESENTED IN TABLE 3. WHEN THE SUPERNATANT FRACTION OF EMBRYONIC CHICK LIVER WAS INCUBATED AT pH 5.2 WITH α-KETOGLUTARATE AND DPNH, NO GLUTAMATE

TABLE 2. RESPONSE OF TRUE AND ARTIFACTUAL GLUTAMIC DEHYDROGENASE ACTIVITY TO KNOWN INHIBITORS AND ACTIVATORS.

FRACTION	pH	ADDITION	SPECIFIC ACTIVITY (AVERAGE)*
MITOCHONDRIA (Mw)	7.6	-	540
MITOCHONDRIA (Mw)	7.6	5 x 10^{-5}M DES	0
MITOCHONDRIA (Mw)	7.6	1 x 10^{-3}M EDTA	580
MITOCHONDRIA (Mw)	7.6	5 x 10^{-4}M ADP	1060
MITOCHONDRIA (MD)	7.6	-	2450
MITOCHONDRIA (MD)	7.6	5 x 10^{-5}M DES	0
MITOCHONDRIA (MD)	7.6	1 x 10^{-3}M EDTA	7000
MITOCHONDRIA (MD)	7.6	5 x 10^{-4}M ADP	11000
SUPERNATANT	7.6	-	0
SUPERNATANT	5.2	-	320
SUPERNATANT	5.2	5 x 10^{-5}M DES	330
SUPERNATANT	5.2	1 x 10^{-4}M DES	320
SUPERNATANT AT 37°C	5.2	1 x 10^{-4}M DES	320
SUPERNATANT	5.2	1 x 10^{-3}M EDTA	330
SUPERNATANT	5.2	5 x 10^{-4}M ADP	300

* AVERAGE OF DUPLICATE VALUES FROM TWO SEPARATE EXPERIMENTS. THE RESULTS WERE OF THE SAME ORDER FOR 14-DAY EMBRYONIC CHICK LIVER AND LIVER FROM CHICKS ONE DAY AFTER HATCHING. NH_4^+ WAS NOT ADDED TO THE SUPERNATANT FRACTIONS IN THESE EXPERIMENTS.

WAS FORMED. RATHER, THE PRODUCT OF THE REACTION WAS α-HYDROXYGLUTARATE, AS INDICATED BY CHROMATOGRAPHIC DETERMINATIONS ON TWO SUBSTRATA AND WITH TWO SOLVENTS. AFTER INCUBATION FOR SHORTER TIME PERIODS, BOTH α-KETOGLUTARATE AND α-HYDROXYGLUTARATE WERE PRESENT IN THE MIXTURE. THE ABSENCE OF α-HYDROXY-GLUTARATE AFTER INCUBATION WITH BOILED ENZYME EXTRACT SHOWS THAT THE REACTION IS ENZYMATIC. IN PRELIMINARY EXPERIMENTS, NO EVIDENCE WAS OBTAINED FOR THE REVERSE REACTION (WEIL-MALHERBE, 1937), USING α-HYDROXYGLUTARATE AS SUBSTRATE AND DPN, PHENAZINE METHOSULFATE, OR METHYLENE BLUE AS ELECTRON ACCEPTORS AT

pH 5.2 or 7.6. The exact nature of the DPNH: α-ketoglutarate oxidoreductase involved in the present experiments remains to be established.

TABLE 3. R_F values after thin-layer or paper chromatography of chick liver incubation mixtures and of standard compounds.

Sample	Solvent A		Solvent B	
	Silica gel	Paper	Silica gel	Paper
α-Ketoglutarate	0.36, 0.37	0.45	0.19, 0.22	0.30
α-Hydroxyglutarate	0.21, 0.22	0.27	0.09, 0.08	0.12
α-Hydroxybutyrate	0.79, 0.82	0.88	0.25, 0.29	0.41
Glutamate	0.10, 0.13	0.10	0 , 0	0
Glutarate	0.71, 0.79	0.80	0.21, 0.27	0.30
Pyruvate a)	0.43, 0.40	0.52	0.22, 0.22	0.30
b)	0.54, 0.51	0.61		
Malate	0.32, 0.35	0.40	0.16	
Isocitrate	0.36, 0.36	0.48	0 , 0	0
Lactate	0.64, 0.68	0.73	a) 0.59, 0.62	0.61
			b) 0.50, 0.49	0.72
Blank	0.36, 0.36	0.44	0.19, 0.20	0.32
Incubated mixture	0.21, 0.24	0.28	0.11, 0.10	0.13

Solvent A: Butanol-acetic acid-water (12:3:5); Solvent B: Ethanol-ammonia-water (16:1:3). The results of two experiments on silica gel and a single experiment on paper are given for each solvent. Separations were more satisfactory with solvent A. The chromatographic patterns are in essential agreement with the data presented by Smith (1960). The blank consisted of the entire incubation mixture with boiled enzyme extract. Pyruvate and, probably, lactate yielded two separate spots in solvent A and lactate in solvent B. An R_F value of 0 indicates a spot remaining at the origin following chromatography.

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