STUDIES ON A GLUCURONOLACTONE DECARBOXYLASE*, **, ***

JOSEPH L. RABINOWITZ AND THEODORE SALL

Radioisotope Service and Medical Research Laboratory, Veterans Administration Hospital, Philadelphia, Pa. (U.S.A.)

INTRODUCTION

The preparation of a soluble enzyme system capable of decarboxylating ^{14}C-GL was previously reported from this laboratory[1]. This paper will present evidence concerning the cofactor requirements and optimal conditions for this enzyme system obtained from rat kidney. By the use of substrates labeled exclusively in the 6 position, it was possible to demonstrate that the resulting CO_2 is derived predominantly from this carbon. Similar results were obtained with GL, GLA or the sodium salts[1]. These findings differ in some respects from those reported by EISENBERG[2] and may suggest that a different reaction mechanism is involved. Several pathways involving the metabolic conversion of GL to L-xylulose and D-ribose have been proposed (ENKLEWITZ[3], TOUSTER[4], HORECKER[5]). Definitive identification of such metabolic products should clarify the reaction mechanisms and the intermediates involved.

EXPERIMENTAL

Male Wistar rats (100–150 g) were sacrificed by cervical fracture, the kidneys removed and placed in a cold buffered solution (K_2HPO_4, 0.067 M; KH_2PO_4, 0.042 M; $MgCl_2$, 0.06 M; nicotinamide 0.03 M, pH 7). All the preparative enzyme work was carried at 0–1° C. Cell-free homogenates were prepared from kidney minces in two and a half volumes of buffer by homogenizing for 30 seconds in a loose-fitting Potter-Elvehjem glass homogenizer. Cell debris and nuclei were removed from the homogenate by centrifugation at 2000 r.p.m. for eight minutes. The supernatant fraction was centrifuged at 36,000 r.p.m. for 30 minutes (Spinco preparative centrifuge). The resulting clear particle-free supernatant fluid was the preparation utilized throughout these investigations. Prior to assay the enzyme system was fortified with cofactors. The cofactor concentrations used were those found to be optimal for 10 mg of GL. Approximately 0.3 mg each of ATP, DPN, UTP, and TPP were added to a 1 ml solution of particle-free enzyme system (final volume 3 ml). These amounts of cofactors were used throughout, except when otherwise specified.

The incubation temperature was 37° C except when specified. During the incubations the flasks were gently shaken in an atmosphere of carbon dioxide-free oxygen, except when specified. The CO_2 from the decarboxylation reaction was slowly flushed by a stream of oxygen and collected in a saturated barium hydroxide solution layered with toluene. The resulting barium carbonate was washed repeatedly and dried at 120° C for 16 hours. The powdered material was suspended in acetone, transferred to pre-weighed steel planchets and assayed after subsequent drying with a flow counter to a statistical error of $\pm$ 3%. The c.p.m. obtained were corrected to infinite thinness and are reported as c.p.m./mg of carbon.

* The radioactive materials were obtained on allocation of the U.S. Atomic Energy Commission.

** The following symbols are used throughout this paper: GL = glucuronolactone; GLA = glucuronic acid; AMP = adenosine-5′-monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; UMP = uridine monophosphate; UDP = uridine diphosphate; UTP = uridine triphosphate; TPP = thiamine pyrophosphate; c.p.m. = counts per minute.

*** Aided by a grant from the Muscular Dystrophy Associations of America, Inc.

References p. 294.

TABLE

DECARBOXYLATION OF UNIFORMLY

*Substrate***	*Weight of added substrate (mg)*	*Radioactivity introduced (c.p.m.)*			
		Specific activity per mg carbon		*Total activity in total sample*	
		all carbons	*in carbon-6*	*all carbons*	*in carbon-6*
GL-^{14}C multi-labeled	2.5	76,000	76,000 (calculated)	76,000	12,660
GL-^{14}C multi-labeled	5.0	76,000	76,000 (calculated)	152,000	25,330
GL-6-^{14}C	2.5	57,000	342,000	57,000	57,000
GL-6-^{14}C	5.0	57,000	342,000	114,000	114,000

* Corrected for endogenous CO_2 in parallel runs without substrate.
** To enzyme system were added 0.3 mg each of ATP, DPN, UTP, TPP, and the radioactive substrat
*** % yield of theory (wt.).

The substrates were assayed for radioactivity after oxidation to barium carbonate. The washed barium carbonate was collected in pre-weighed centrifuge tubes, for gravimetric measurements. Most experiments were carried out in duplicate or triplicate.

In order to determine the cofactor requirements, the enzyme preparations were pre-treated with charcoal for 3–5 minutes (10 mg of charcoal for 1 ml of the enzyme preparation). The preparation was freed of charcoal by centrifugation at 2000 r.p.m. for 8 minutes.

RESULTS

The evidence indicates that is is predominantly carbon-6 of GL and GLA which is decarboxylated by the rat kidney enzyme system.

Table I presents the results obtained when multiply-labeled and 6-^{14}C-glucuronolactone were employed as substrates. The recovered CO_2 had essentially the same specific radioactivity as carbon-6 in all cases. The total recovery of CO_2 or total activity recovered was usually 10–11% (by gravimetric and radioactive measurements). Serial additions of the enzyme increased the yield, approximately 10% for each successive addition. That these systems were saturated with respect to the enzyme system is indicated by the fact that the yields were not increased by further addition of substrates.

TABLE II

DECARBOXYLATION OF 1-^{13}C AND 6-^{13}C-GL

*Substrate**	*Weight in mg*	*% excess in ^{13}C substrate*	*% excess in ^{13}C recovered CO_2***
Glucuronolactone	5	0	0.0
Glucuronolactone 1-^{13}C	5	20	0.6
Glucuronolactone 6-^{13}C	5	20	16.7

* Additions were 0.3 mg each of ATP, DPN, UTP, TPP (final volume 3 ml); incubation 1 ½ hours at 37°, gas phase O_2.
** Corrected for weight of endogenous CO_2 in parallel run without substrates.

References p. 294.

I

AND 6-^{14}C-LABELED GL SUBSTRATES

Radioactivity recovered (c.p.m.)					Recovered CO_2 (% yield)*, ***	
		% of				
Specific activity* per mg C	Total activity in total CO_2	Original specific activity if only carbon-6 is involved	Total activity		if all carbons are involved	if only carbon-6 is involved
			if all carbons are involved	if only carbon-6 involved		
61,510	1,120	81	1.5	8.8	1.8	10.8
63,990	2,250	85	1.5	8.9	1.7	10.5
358,650	6,300	104	—	11.0	1.7	10.5
321,950	11,900	95	—	10.5	1.8	11.0

(final vol. 3.3 ml); incubation for 1 ½ h at 37° gas phase O_2.

Table II presents additional evidence that carbon-6 is predominantly involved; in this instance 1-^{13}C and 6-^{13}C-GL were employed.

Maximal values were obtained when the enzyme system was incubated at 37°C (see Table III).

TABLE III

EFFECT OF TEMPERATURE ON THE ENZYME SYSTEM*

Temperature	Recovered radioactivity in $BaCO_3$ c.p.m./mg C
15°	3,000
26°	11,000
37°	15,000
46°	7,000
56°	6,000
100°	200

* Additions were 0.3 mg each of ATP, DPN, UTP, TPP and multiply labeled GL (final volume 3 ml). Incubation with gentle shaking for 1 ½ h, gas phase O_2.

Different cofactors (AMP, ADP, ATP, UMP, UDP, UTP, DPN, TPN and TPP) were individually tested for their effects on the enzyme system. Optimal results were obtained with a combination of ATP, UTP, DPN and TPP, as is shown in Table IV.

The effect of each individual cofactor in the presence of optimal quantities of the others is illustrated in Fig. 1.

The requirement for the nicotinamide employed in the buffer during the preparation of the enzyme system and the requirement for oxygen are demonstrated in Table V.

Mg^{++} or Mn^{++} ions are required (Fig. 2).

The pH optimum was found[1] to be 7 (Fig. 3).

References p. 294.

TABLE IV

(1) Enzyme	(2) AMP	(3) ADP	(4) ATP	(5) DPN	(6) TPN	(7) UMP	(8) UDP	(9) UTP	(10) TPP	(11) c.p.m./mg C
+	+			+		+				972
+	+				+	+				701
+	+			+			+			1,736
+	+				+		+			1,135
+	+			+				+		4,267
+	+			+				+	+	4,775
+	+				+			+		1,995
+	+				+			+	+	2,269
+		+		+		+				580
+		+			+	+				789
+		+		+						1,937
+		+			+		+			2,018
+		+		+				+		4,088
+		+		+				+	+	6,400
+		+			+			+		1,759
+		+			+			+	+	2,068
+			+	+		+				1,350
+			+		+	+				1,937
+			+	+			+			1,688
+			+		+		+			1,503
+			+	+				+		6,656
+			+	+				+	+	11,760
+			+		+			+		4,689
+			+		+			+	+	6,972
			+	+				+	+	40
+										489

(1) Enzyme, charcoal-treated; (2) AMP 1.2 μM per 3.0 ml system; (3) ADP 1.2 μM per 3.0 ml system; (4) ATP 1.2 μM per 3.0 ml system; (5) DPN 1.0 μM per 3.0 ml system; (6) TPN 1.0 μM per 3.0 ml system; (7) UMP 1.3 μM per 3.0 ml system; (8) UDP 1.3 μM per 3.0 ml system; (9) UTP 1.3 μM per 3.0 ml system; (10) TPP 1.8 μM per 3.0 ml system; (11) Recovered radioactivity in CO_2.
Substrate 1.2 mg of uniformly labeled ^{14}C-GL. Incubation time 1.5 h, 37° C, gas phase O_2.

TABLE V

EFFECT OF GAS PHASE AND NICOTINAMIDE ON ENZYME SYSTEM*

Enzyme system	Gas phase	Nicotinamide	Radioactivity recovered c.p.m.
Kidney enzyme system	O_2	+	10,000
Kidney enzyme system	O_2	—	2,510
Kidney enzyme system	N_2	+	2,550
Kidney enzyme system	N_2	—	1,340

* Additions were 0.3 mg each of ATP, DPN, UTP, TPP and ^{14}C-GL uniformly labeled; 1 ½ h incubation at 37° C (final volume 3 ml).

The effect of varying the concentration of substrate is illustrated in Fig. 4.

A 10 minute period of activation is required for the decarboxylation reaction. During the subsequent 20 minutes the reaction proceeeds at an increased rate; after 30 minutes the reaction rate was constant (Fig. 5).

References p. 294.

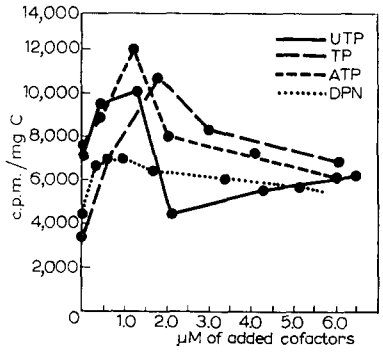

Fig. 1. Cofactor effects. To 1 ml of enzyme system (charcoal-treated). Additions of 1 mg of all other cofactors except the one under investigation. Substrate 1.2 mg ^{14}C-GL (final volume 3.9 ml); gas phase oxygen; incubated 1 ½ h at 37° C.

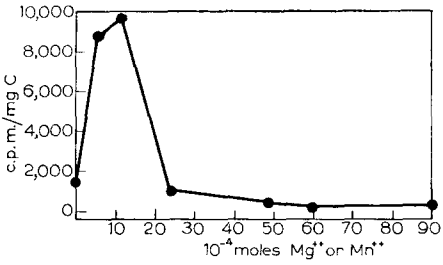

Fig. 2. Effect of Mg^{++} and Mn^{++}. Additions were 0.3 mg each of ATP, DPN, UTP, TPP and ^{14}C-GL. Enzyme system 2 ml (final volume 3 ml); incubated 1 ½ h at 37° C; gas phase oxygen.

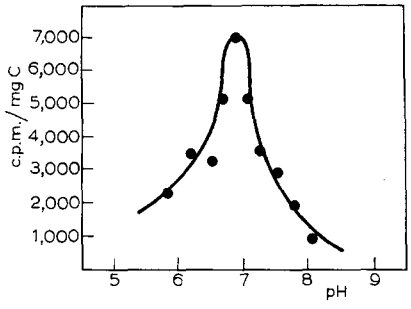

Fig. 3. Change in enzyme activity with pH. Additions were 0.3 mg each of ATP, DPN, UTP, TPP and ^{14}C-GL. Incubated 1 ½ h at 37° C; gas oxygen (final volume 3 ml). The phosphate buffer was varied for pH, but kept at the same ionic strength. Mg and nicotinamide remained constant.

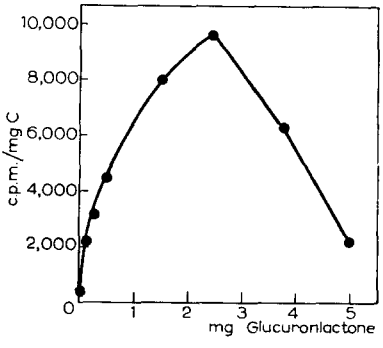

Fig. 4. Effect of substrate concentration. Additions were 0.3 mg each of ATP, DPN, UTP and TPP (final volume 3 ml). Incubated 2 h at 37° C; gas phase oxygen.

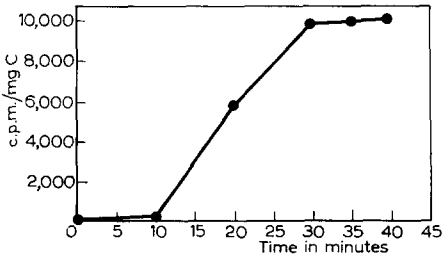

Fig. 5. Time curve. Additions were 0.3 mg of ATP, DPN, UTP, TPP and ^{14}C-GL (final volume 3 ml). Gas phase oxygen. Incubation at 37° C.

References p. 294.

DISCUSSION

EISENBERG AND FIELD[6] reported the failure of rat liver homogenates to decarboxylate GL at an appreciable rate. DOUGLAS AND KING[7] indicated that the intact animal is capable of decarboxylating GL. Evidence is presented in this paper for the existence of this decarboxylase in *in vitro* systems. With the fortified enzyme system, average yields of 10–12% of CO_2 were obtained. Further additions of fresh enzyme resulted in additional formation of CO_2. The previous suggestion[1] that carbon-6 is probably the only carbon involved in this decarboxylation is corroborated by the use of both ^{13}C- and ^{14}C-labeled substrates. The total contribution of the other carbons was negligible.

Attempts to determine whether GL must first be converted to GLA prior to decarboxylation were unsuccessful. Parallel and repeated runs did not consistently show significant differences between the rates of decarboxylation fo GL and GLA. Similar results were obtained whether fresh[1], aged or pre-heated enzyme preparations were employed.

It is possible that the crude preparation contains different enzymes capable of utilizing the substrates to form different products. The actual liberation of CO_2 may be the terminal step of a series of different reactions, resulting from the action of these enzymes[8]. This could also account for the contribution of other carbon atoms to the CO_2.

Experimental evidence indicates that the optimal activity for this enzyme system was obtained with a combination of ATP, DPN, UTP and TPP, although AMP, ADP, ATP, DPN, TPN, UDP, UTP and TPP singly stimulate the reaction rate when compared with an unfortified control.

Enzyme purification and isolation of resulting labeled intermediates are currently under investigation in the hope that further information will be obtained on the mechanism of these reactions.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Dr. S. GURIN for his encouragement and advice. Through the generosity of Doctors J. ISBELL, F. EISENBERG and N. E. ARTZ ^{14}C- and ^{13}C-GL and related carbohydrates were procured. Dr. S. WEINHOUSE generously aided with mass spectrometric analysis. The technical assistance of Misses O. OLEKSYSHYN, E. C. FISHER and H. RICHARDS is acknowledged.

SUMMARY

1. A particle-free enzyme system of rat kidney has been prepared which is capable of decarboxylating GL and GLA. When fortified with UTP, TPP, DPN, ATP and Mg^{++} maximal effects are obtained. The reaction is aerobic.

2. The enzyme system yields approximately 12% of CO_2 which is predominantly derived from carbon-6 of GL and GLA. Increased yields may be obtained by further addition of enzyme.

REFERENCES

1 J. L. RABINOWITZ, *J. Am. Chem. Soc.*, 77 (1955) 5180.
2 F. EISENBERG AND J. B. FIELD, *Federation Proc.*, 15 (1956) 247.
3 M. ENKLEWITZ AND M. LASKER, *J. Biol. Chem.*, 110 (1935) 443.
4 O. TOUSTER, R. M. HUTCHESON AND L. RICE, *J. Biol. Chem.*, 215 (1955) 677.
5 B. L. HORECKER, P. Z. SMYRNIOTIS AND J. E. SEEGMILLER, *J. Biol. Chem.*, 193 (1951) 383.
6 F. EISENBERG AND J. B. FIELD, *J. Biol. Chem.*, (in press).
7 J. R. DOUGLAS AND C. G. KING, *J. Biol. Chem.*, 203 (1953) 889.
8 J. J. BURNS, C. EVANS AND F. M. MOSBACH, *Federation Proc.*, 15 (1956) 406.

Received June 16th, 1956