Oxidative decarboxylation of retinoic acid in microsomes of rat liver and kidney

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ABSTRACT Liver and kidney microsomes have been found to catalyze a rapid decarboxylation of retinoic acid in vitro. The reaction requires NADPH and Fe^{2+}, and is further stimulated by the presence of pyrophosphate. Thiamine pyrophosphate contained sufficient iron as an impurity to provide strong enhancement of the reaction in the absence of added iron. The decarboxylation could also be shown to occur nonenzymatically in the presence of ascorbate, Fe^{2+}, and boiled microsomes, but there was little autoxidation resulting in decarboxylation. The reaction was strongly inhibited by chelating agents, *N,N'*-diphenyl-*p*-phenylene diamine, phenazine methosulfate, and ferricyanide, and resembled lipid peroxidation in both its cofactor requirements and response to inhibitors.

The product of the reaction appeared to lack only the C-15 of the original retinoic acid molecule. It was not retained by diethylaminoethyl cellulose, was more polar than retinoic acid upon silicic acid chromatography, had a lower UV absorption maximum (295 mμ) than the starting product, and seemed to have an aldehyde group at C-14.

The physiological significance of the decarboxylation remains to be assessed, but its rapidity makes it important to in vitro work on retinoic acid.

KEY WORDS retinoic acid · decarboxylation · peroxidation · rat · microsomes · inhibition · chelation · iron

RETINOIC ACID HAS BEEN SHOWN to be decarboxylated by rats in vivo (1, 2) and by liver and kidney slices in vitro (3). The physiological importance of this decarboxylation in relation to the action of vitamin A is unknown, but Yagishita, Sundaresan, and Wolf (4) have reported finding a metabolite of retinoic acid that had growth-promoting activity and that was formed in vivo by loss of at least the terminal carbon atom. We describe here a system, located in the microsomal fraction of rat liver and kidneys, that catalyzes rapid decarboxylation of retinoic acid. The microsomal reaction differs from that of the previously described microsomal hydroxylases (5–7); it is probably initiated by the pyridine nucleotide enzyme system that catalyzes the peroxidation of microsomal lipids (8–13).

Abbreviations: TPP, thiamine pyrophosphate chloride; DPPD, *N,N'*-diphenyl-*p*-phenylene diamine; PP_i, sodium pyrophosphate, DEAE, diethylaminoethyl.

EXPERIMENTAL METHODS

Retinoic acid was obtained from Distillation Product Industries, Rochester, N. Y. Hoffmann-La Roche and Co., Basel, Switzerland generously provided retinoic acids-15-^{14}C and -6,7-^{14}C; retinoic acid-14-^{14}C was obtained from Tracerlab Div., Waltham, Mass. All the radioactive retinoic acids were mixed with unlabeled carrier and purified by thin-layer chromatography (3). Thiamine pyrophosphate chloride, A grade (TPP) was obtained from Calbiochem, Los Angeles, Calif.

Tissues were obtained from male rats weighing 250–300 g (Holtzman Company, Madison, Wis.). Microsomes were prepared according to the method of Ernster, Siekevitz, and Palade (14). The final microsomal pellet was rehomogenized in a volume of 0.25 M sucrose equal to that of the original 10% homogenate of tissue. This microsomal suspension was stored in 10-ml portions at −17°C for periods up to 3 wk and thawed as needed.

Incubation Conditions

Retinoic acid was prepared as an aqueous solution of the sodium salt (3) and all incubations were carried out in 25-ml Erlenmeyer flasks in a 3 ml volume. Unless otherwise stated, the final concentration of all components was as follows: retinoic acid, 3.3 μM; TPP, 3.6 mM;

KCl, 50 mM; $MgCl_2$, 5 mM; phosphate buffer (pH 7.3), 5 mM; NADP plus NADPH, 88 μM. The NADPH was provided by a generating system of glucose-6-phosphate dehydrogenase, NADP, and glucose-6-phosphate, the last two substances having final concentrations of 88 μM and 1 mM, respectively. $^{14}CO_2$ was collected, when necessary, as previously described (3) except that the trapping solution was in the polypropylene center well from the start of the incubation. Flasks were at 0°C at the start and the incubations were carried out at 30°C in a shaker bath either for 20 min (at which time the reaction was complete) or for shorter times as specifically noted in the text. Blanks using heated microsomes or the complete system minus NADPH were included in every experiment, and each value shown represents the average of at least six determinations. The degree of reaction is expressed as the percentage of ^{14}C from retinoic acid-15-^{14}C that was recovered as $^{14}CO_2$. All results shown were obtained with liver microsomes unless stated otherwise.

Chromatography of the Reaction Products

Samples to be chromatographed were incubated under conditions identical with those described above except that 60-ml incubations were carried out in 500-ml Erlenmeyer flasks. The reaction was allowed to proceed for only 3–4 min, at which time 75 ml of cold (0°C) chloroform, containing 100–300 μg of carrier retinoic acid and 10 mg of α-tocopherol, and 150 ml of cold methanol were added. The extraction was carried out in the cold under N_2 according to the procedure of Bligh and Dyer (15). The chloroform phase was evaporated to dryness under reduced pressure and the residue was dissolved in 1.0 ml of absolute ethanol. DEAE-cellulose (Bio-Rad Laboratories, Richmond, Calif.) and silicic acid were prepared as previously described (16, 17). The ethanol solution of the chloroform extract was applied to the DEAE-cellulose column. The nonacidic fractions eluted from this column were combined and dried, and the residue was taken up in Skellysolve B (petroleum ether, bp 60–65°C) and applied to a 15 g multibore column of silicic acid in Skellysolve B. The mixture was chromatographed in a hyperbolic gradient elution system (18). Fractions were counted in a Packard model 314 EX liquid scintillation counter.

Tollens' Oxidation and Schmidt Degradation

The material to be oxidized was dissolved in 0.5 ml of ethanol to which we added 1 ml of Tollens' reagent (10% $AgNO_3$ mixed with an equal volume of 10% NaOH, and the precipitate just dissolved by dropwise addition of dilute NH_3). The mixture was heated to 50°C for 2 min, allowed to stand for 2 min, and then extracted twice with diethyl ether (5 ml). After acidification with concentrated HCl, the ether extraction was repeated twice. All ether extracts were washed to neutrality. The successive ether extracts from either acidic or basic solutions were combined, dried under N_2, and taken up in ethanol for chromatography on DEAE-cellulose.

The compounds to be subjected to Schmidt degradation were dried under N_2 in a 25 ml Erlenmeyer flask. First 0.4 ml of concentrated sulfuric acid and then 50 mg of NaN_3 were added to the flask at 0°C. The flask was sealed with a rubber septum stopper containing a CO_2 trap (3), heated slowly to 60°C for 10 min with constant swirling, and shaken in a water bath at 65°C for 1 hr. After an additional hour at room temperature, the CO_2 traps were removed to be counted, and 2 ml of 50% ethanol was added to the solution remaining in the flask. Three 0.5 ml aliquots were counted.

Iron Analyses

Analyses for non-heme iron were done by the method of Ramsay (19).

RESULTS

The requirements for the microsomal decarboxylation of retinoic acid are shown in Table 1. There was little autoxidative decarboxylation of the retinoic acid in the absence of microsomes or in the microsomal preparation with no added cofactors. The addition of Fe^{2+} provided

TABLE 1 REQUIREMENTS FOR THE DECARBOXYLATION OF RETINOIC ACID IN LIVER MICROSOMES

Addition to Reaction System*	% of Control†		
		20 min Reaction	
	2–3 min Reaction	Untreated Microsomes	Heated Microsomes
−Microsomes	12	7	
None	12	9	7
+Fe^{2+}	12	19	
+NAPDH	32	58	
+TPP		10	
+NADPH + Fe^{2+}	65	86	
+NADPH + TPP	100†	100†	5
+NADPH + PP_i	59		
+NADPH + TPP + Fe^{2+}	90	101	
+NADPH + PP_i + Fe^{2+}	117	105	
+Ascorbate	23	51	
+Ascorbate + Fe^{2+}	53	87	79
+Ascorbate + Fe^{2+} + TPP	92	98	
+Ascorbate + Fe^{2+}, −microsomes		6	

* Present in all systems were KCl, $MgCl_2$, PO_4 (pH 7.3), microsomes, and retinoic acid-15-^{14}C as stated in the Methods section. When added, NADPH was provided by the generating system described. The final concentrations of added components were Fe^{2+}, 10 μM except in ascorbate systems where it was 200 μM; TPP, 3.6 mM; PP_i, 0.3 mM; and ascorbate 1 mM.

† For the 2–3 min reaction 0.2 ml of microsomes was used and the average control was 20% decarboxylation; for the 20-min reactions 0.5 ml was used and the control value was 48%. The control was chosen as the system with NADPH and TPP added.

no stimulation of the reaction unless NADPH was present, whereas there was a slight reaction in the presence of NADPH alone, probably due to the presence of some non-heme iron in the microsomes. The addition of iron to the microsomal system containing NADPH more than doubled the rate of retinoic acid decarboxylation, and this rate was redoubled by the addition of inorganic pyrophosphate (PP_i). Only in the complete system containing Fe^{2+}, NADPH, and pyrophosphate did the decarboxylation occur to the maximum extent. TPP also enhanced the decarboxylation. Although the results seemed to indicate that TPP obviated the requirement of the reaction for Fe^{2+}, analysis showed that the TPP (Calbiochem, lot 62245) was contaminated with 246 ng of iron per mg. The PP_i contained only 5 ng per mg. Examination of Fig. 1, which illustrates the dependence of the reaction on the concentration of added Fe^{2+}, shows that a concentration of 7.3 μM iron, which would result from 3.6 mM TPP, would largely fulfill the iron requirement, whereas 0.01 μM iron resulting from 0.3 mM PP_i would not. Similarly, Hochstein, Nordenbrand, and Ernster (9) have reported finding enough iron contamination in commercial ADP to overcome the iron requirement for peroxidation of microsomal lipids.

Enhancement of the reaction by various levels of TPP, ATP, and PP_i is illustrated in Fig. 2. Each of these reagents stimulated the decarboxylation; PP_i produced the maximum effect at a much lower concentration than did either the TPP or the ATP. These data were obtained at 12 min reaction time; although the maxima might have been sharper at shorter times, the general shape of the curves would have been unchanged.

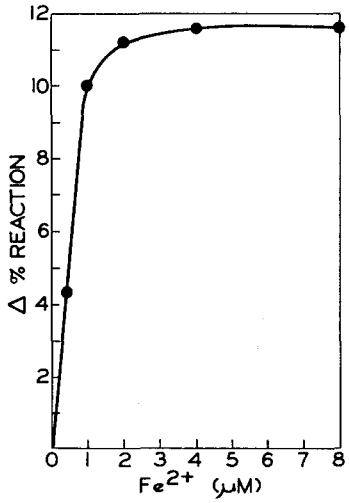

FIG. 1. Effect of various concentrations of Fe^{2+} on the decarboxylation of retinoic acid in rat liver microsomes. The reaction system was as described in Table 1 with an NADPH-generating system added. The ordinate represents the difference in the percentage of decarboxylation with and without Fe^{2+} at the concentration indicated. Each flask contained 0.5 ml of microsomes and the reaction was carried out for 20 min.

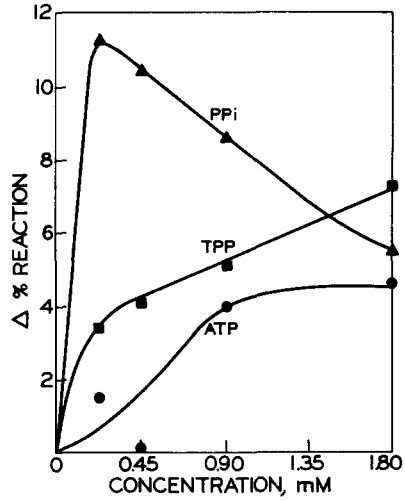

FIG. 2. Effects of ATP, TPP, and inorganic pyrophosphate on the reaction. The reaction system was as described in Table 1. NADPH, supplied as a generating system, and Fe^{2+} (10 μM) were in all flasks, and ATP (●), TPP (■), or PP_i (▲) was added in the concentrations indicated. The percentage reaction for the system lacking pyrophosphate has been subtracted from all values. Each flask contained 0.5 ml of microsomes and the reaction was carried out for 20 min.

Table 2 shows that retinoic acid could also be decarboxylated by a nonenzymatic reaction that utilizes Fe^{2+} and ascorbate (8, 11, 20). This reaction occurred with heated microsomes as well as with untreated microsomes, whereas the enzymatic NADPH-linked reaction did not. In the absence of any microsomal lipid, however, there was little autoxidative decarboxylation of the retinoic acid. Neither this reaction system nor the NADPH-dependent system released any $^{14}CO_2$ from retinoic acids-14-^{14}C or -6,7-^{14}C under any conditions.

Table 2 shows that retinoic acid was decarboxylated in rat kidney microsomes to about the same extent as in liver microsomes. That the value for the reaction in the presence of NADPH alone was significantly lower in the kidney than in the liver microsomes possibly relates to the lower non-heme iron content of the kidney microsomes (unpublished results).

TABLE 2 DECARBOXYLATION REACTION IN KIDNEY AND LIVER MICROSOMES

Addition to Reaction System*	% Decarboxylation	
	Liver	Kidney
None	4.0	1.9
+NADPH	28.8	13.8
+NADPH + Fe^{2+}	42.7	35.8
+NADPH + TPP	47.7	40.5
+NADPH + TPP + Fe^{2+}	48.3	40.6

* The reaction system and the concentrations of Fe^{2+} and TPP were as described in Table 1. Each flask contained 0.5 ml of microsomes and the reaction was allowed to proceed to completion (20 min reaction).

Kinetics

Fig. 3 shows that the oxidative decarboxylation of retinoic acid was a rapid reaction. We obtained these figures by periodically removing and counting a 0.2 ml aliquot of the incubation medium, thus monitoring the decrease in the radioactivity of the medium. This method correlated quite well with the $^{14}CO_2$ measurements otherwise used to assay this reaction, though it was subject to considerably greater statistical error.

In Fig. 4 the degree of completeness of the reaction in 1.5 min has been plotted versus microsomal concentration. When the reaction was allowed to go to completion (30 min), all levels of microsomes except the 0.1 ml level catalyzed the same degree decarboxylation. Possibly other nondecarboxylative reactions of the retinoic acid compete more favorably as the rate of decarboxylation decreases. The results also show that storage of the microsomes at 4°C for 2 days decreased the activity to approximately half that of fresh preparations.

Fig. 5 shows that the amount of $^{14}CO_2$ produced at the completion of the reaction was directly proportional to the amount of retinoic acid in the 0–12 μg range. Studies with very short incubation times, which approximate initial velocity studies, did show the expected rapid increase and leveling off of reaction rate as the retinoic acid concentration approached saturation.

Inhibitors of the Reaction

The effects of various inhibitors on the reaction are shown in Table 3. The reaction could be completely inhibited by EDTA (a chelating agent), by phenazine methosulfate or ferricyanide (acceptors for microsomal NADPH-linked flavoprotein dehydrogenase), or by DPPD (an inhibitor of peroxidations [5, 8]). SK&F-525A (β-diethyl-aminoethyl diphenylpropyl acetate), an inhibitor of both peroxidation and microsomal hydroxylation (5, 8), and *o*-phenanthroline (11) produced effects on this decarboxylation similar to those reported in the literature for lipid peroxidation. The addition of either

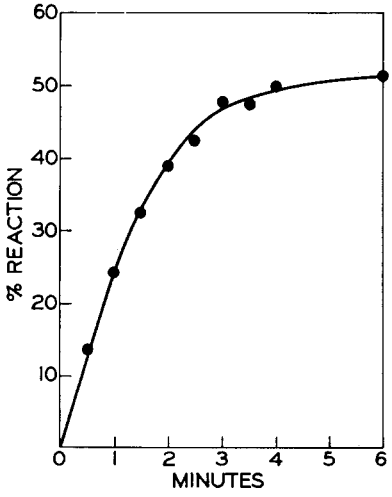

FIG. 3. Rate of microsomal decarboxylation of retinoic acid. The reaction system was as described in Table 3 except that the flasks contained 0.3 ml of fresh microsomes and the progress of the reaction was measured by the decrease in the radioactivity of the medium, not by $^{14}CO_2$ collection and counting.

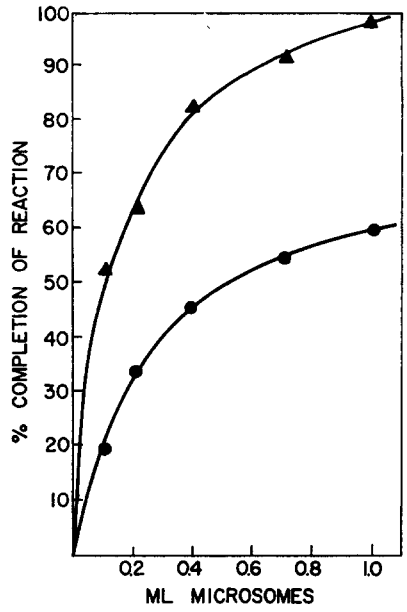

FIG. 4. Effect of varying the amount of microsomes on the rate of the decarboxylation. The reaction system was as described in Table 3. Each flask contained the amount of microsomes specified, either freshly prepared (▲) or aged at 4°C for 48 hr (●). The reaction was stopped after 1.5 min. The amount of decarboxylation in 30 min was chosen as the completed reaction.

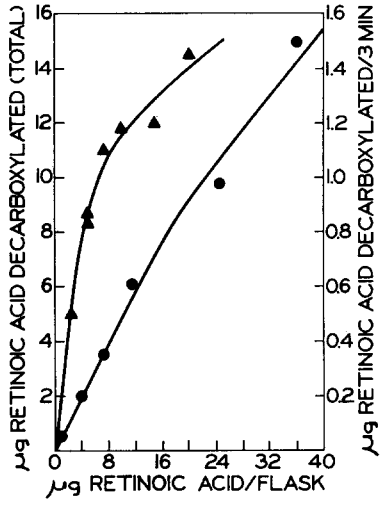

FIG. 5. Effect of concentration of retinoic acid on the initial velocity and the completed microsomal decarboxylation. The reaction system was as described in Table 3. Retinoic acid-15-^{14}C was added in the amounts indicated. The complete reaction was either assayed at 20 min (●), in which case 0.5 ml of microsomes was used, or measured at 3 min (▲), in which case only 0.25 ml of microsomes was used.

TABLE 3 INHIBITORS OF MICROSOMAL DECARBOXYLATION OF RETINOIC ACID

Inhibitor	Concentration	% of Control
	mM	
NaCN	1.3	77
NaN_3	1.3	100
p-Chloromercuribenzoate	0.7	39
$K_3Fe(CN)_6$	0.2	0
Phenazine methosulfate	0.02	0
EDTA	0.2	0
	0.01	95
NADP–NADPH 10:1*	0.9	48
Phenobarbital	5	72
Aminopyrine	10	39
	5	63
DPPD	1.3×10^{-4}	0
SK&F-525A†	0.5	13
	0.1	59
o-Phenanthroline	1.0	30
$CO–O_2$ 2:1		100

The system contained KCl, $MgCl_2$, PO_4 (pH 7.3), TPP, and retinoic acid-15-^{14}C as stated in the Methods section. NADPH was provided by the generating system described. The reaction was analyzed after 2–3 min, at which time 20–40% decarboxylation had occurred. Each flask contained 0.2 ml of freshly prepared microsomes which were preincubated with the inhibitor for 15 min at 0°C.

* No generating system was used. An appropriate control was used.

† Obtained by courtesy of Smith, Kline, & French Laboratories, Philadelphia, Pa.

phenobarbital or aminopyrine, drugs which are actively hydroxylated and demethylated by microsomal enzyme systems (21, 22), inhibited this reaction, probably by competition for a common NADPH-oxidizing enzyme (23). Carbon monoxide, which strongly inhibits the hydroxylase reaction (6), had no effect on this reaction. NADP is a competitive inhibitor with respect to NADPH (21) and inhibited this reaction as expected. Finally, azide had no effect and cyanide little effect on the reaction, although *p*-chloromercuribenzoate was inhibitory.

The pH curve for the decarboxylation showed a maximum near pH 7.5.

Product of the Decarboxylation

It was possible to monitor the progress of the microsomal decarboxylation spectrophotometrically, since retinoic acid absorbs maximally at 350 mμ. A Beckman DB-G grating spectrophotometer with programmer was used for these studies. The $E^{1\%}_{1cm}$ of retinoic acid in 5 mM phosphate buffer, pH 7.3, was 1150 at a concentration of 1 μg/ml and decreased to 920 at a concentration of 5 μg/ml. When the kinetics of decarboxylation were studied, the contents of the reference cell were identical with those in the sample cell, except that water was substituted for the retinoic acid solution. A high level of glucose-6-phosphate dehydrogenase was used in both cells so that any NADP formed in the reaction would be immediately reconverted to NADPH. A straight baseline was observed for 15 min when both cuvettes contained the complete system minus retinoic acid.

The addition of retinoic acid to the sample cell in the presence of the complete system containing NADPH, Fe^{2+}, and PP_i produced striking spectral changes as the reaction progressed. Fig. 6 shows the gradual appearance of a peak at 295 mμ, which accompanied the decrease in absorption at 345 mμ. The 295 mμ absorption reached a maximum after 9 min reaction and thereafter absorption at all wavelengths in the 280–380 mμ range decreased. A similar spectral change accompanied the reaction in the absence of pyrophosphate. However, the reaction rate was considerably slower and the absorption never reached as high a maximum in the 290–300 mμ region as in the pyrophosphate-stimulated system. It was impossible to observe the scan of the NADPH–TPP system since TPP at the concentration employed had too strong an absorption below about 300 mμ. However, the observable portion of the scan agreed closely with that of the Fe^{2+}–pyrophosphate system. When kidney microsomes were used, reaction took place in all systems studied, although a higher concentration of microsomes was required. The products formed appeared to be identical to those resulting from the reaction with liver microsomes, but the rate of reaction was considerably slower.

To ascertain that the appearance of a product absorbing at 295 mμ could be related to the oxidative decarboxylation of retinoic acid, we carried out several controls. Table 4 shows that the appearance of the 295 mμ absorption required NADPH, Fe^{2+}, and PP_i, and did not occur in the presence of DPPD, a strong inhibitor of the decarboxylation.

An attempt was made to learn more about the product of the reaction. The results of solvent partitioning in the

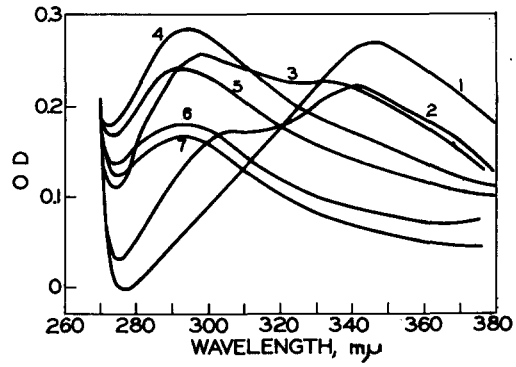

FIG. 6. Spectral changes accompanying the decarboxylation of retinoic acid by liver microsomes. The reaction system was as described in Table 1 except that Fe^{2+} (10 μM), PP_i (0.45 mM), and an NADPH-generating system were added. The retinoic acid concentration was 9.2 μM, and 0.2 ml of microsomes was used in each 3.0 ml cuvette. The reference cell contained no retinoic acid. Repetitive scans were made every 3 min and are numbered 1–7.

TABLE 4 REQUIREMENTS FOR SPECTRAL CHANGES ACCOMPANYING DECARBOXYLATION IN LIVER MICROSOMES

System	% Decrease in OD at 350 mμ in 12 min	% Increase in OD at 295 mμ in 12 min
Complete	50	200–350
−Microsomes	12	0
−Glucose-6-phosphate dehydrogenase	19	0
$-Fe^{2+}$-PP_i	19	0
+DPPD (2.6×10^{-7}M)	3	0

The reaction system was as described in Fig. 6.

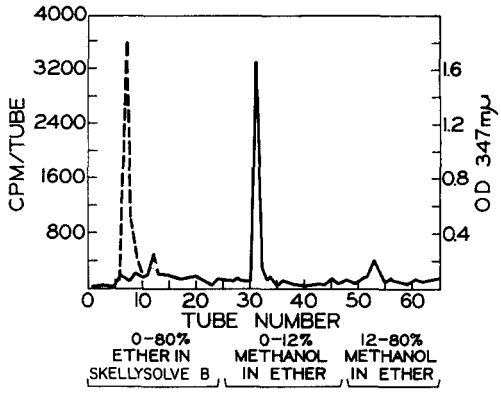

FIG. 7. Silicic acid chromatography of the nonacidic fraction of the chloroform phase after decarboxylation of retinoic acid-14-^{14}C. The solid line represents the cpm/tube; the broken line represents the OD at 347 mμ due to added retinoic acid. Tube 31 was eluted with 6% methanol in ether.

chloroform–methanol–water system of Bligh and Dyer (15) are shown in Table 5. The radioactivity from retinoic acid labeled in the 15, 14, or 6 and 7 positions and incubated either in the absence of microsomes or in the presence of microsomes without addition of the other necessary factors was found almost entirely in the chloroform phase. Reaction with NADPH and TPP formed products which could be found in the methanol–water phase and decreased the amount of radioactivity from retinoic acid-15-^{14}C in the chloroform phase relative to that from retinoic acids-14-^{14}C and -6,7-^{14}C. This relative decrease in the recovery of radioactivity from the -15-^{14}C label was restricted to the chloroform phase, which indicates that the decarboxylated product was recovered in that phase. DEAE-cellulose chromatography of the chloroform extract showed that the decarboxylated material was no longer acidic.

In the absence of TPP, the extent of decarboxylation, the formation of water–methanol-soluble products, and the formation of chloroform-soluble, nonacidic compounds were all decreased as expected. The DEAE-cellulose columns were shown to be effective in retaining any added retinoic acid, both by radioactivity measurements and by examination of OD at 350 mμ.

Silicic acid chromatography of the nonacidic fraction from the DEAE-cellulose column gave the pattern presented in Fig. 7. Retinoic acid-14-^{14}C was used as a marker. There were several peaks, all of which were eluted with more polar solvents than are required for the elution of retinoic acid. The sharp peak eluted by the 0–12% methanol gradient represented 40% of the radioactivity on the column, or about 12% of the original.

To test for a C-14 aldehyde in the decarboxylated product, we subjected the nonacidic (not retained by DEAE-cellulose) chloroform-soluble fraction resulting from the reaction of retinoic acid-14-^{14}C to a Tollens' oxidation followed by a Schmidt degradation. The Tollens' oxidation produced acidic products (ether-extract-

TABLE 5 PARTITIONING OF THE REACTANTS AND PRODUCTS OF THE MICROSOMAL DECARBOXYLATION BETWEEN CHLOROFORM AND METHANOL AND ON DEAE-CELLULOSE CHROMATOGRAPHY

	% of the Original Radioactivity					
		Chloroform–Methanol Extraction			DEAE of $CHCl_3$ Phase	
Reaction System	As $^{14}CO_2$	Total Extracted	$CHCl_3$ Phase	Methanol–Water Phase	Non-acid	Acid
Microsomes + NADPH + TPP						
+ Retinoic acid-15-^{14}C	~50	49.4	16.8	32.7	4.2	12.5
+ Retinoic acid-14-^{14}C	0	94.7	56.3	38.4	30.1	21.8
+ Retinoic acid-6,7-^{14}C	0	97.1	61.5	35.6	43.1	11.0
Microsomes NADPH						
+ Retinoic acid-15-^{14}C	~15	84.9	70.3	14.4	2.2	68.0
+ Retinoic acid-14-^{14}C	0	96.0	84.3	11.7	11.1	73.4
NADPH + TPP						
Retinoic acid-^{14}C*	0	99.5	91.0	8.3	8.5	81.5
Microsomes						
Retinoic acid-^{14}C*	0	98.5	87.9	12.1	8.0	80.0

All concentrations and procedures as described in Methods section.
* Each of the labels partitioned very similarly.

able after acidification of the reaction mixture and retained by DEAE-cellulose) which when subjected to the Schmidt degradation were shown to contain more than 80% α-carboxyl, if retinoic acid-15-^{14}C is taken as standard. (The standard, like some other lipid compounds (24), gave only 65% $^{14}CO_2$ in the Schmidt degradation even though butyric acid-1-^{14}C gave nearly 100%.) These results indicate the presence of a 14-aldehyde group which becomes oxidized to 14-carboxyl upon Tollens' oxidation and is then decarboxylated in the Schmidt degradation. Only 4% of the original radioactivity could be characterized as 14-aldehyde by the above procedure, but this is a minimum estimate because of losses incurred in the Tollens' oxidation. The Schmidt degradation does not produce any $^{14}CO_2$ from retinoic acid-14-^{14}C.

DISCUSSION

The results presented in this paper indicate that retinoic acid is capable of undergoing a rapid decarboxylation in vitro in the microsomal fraction of rat liver and kidney. The reaction differed from an NADPH-dependent hydroxylation in that it required Fe^{2+}; could be made to occur nonenzymatically in the presence of ascorbate, Fe^{2+}, and boiled microsomes; was not inhibited by carbon monoxide; and was completely inhibited by DPPD, which does not inhibit the oxidative demethylation of aminopyrine (21). Furthermore, there was no induction of decarboxylation activity following treatment of rats for 4 days with phenobarbital at the level of 100 mg/kg, (unpublished results) which is known to increase the level of the hydroxylating enzymes 3- to 5-fold (5, 22, 25). The reaction did display all the characteristics of NADPH-dependent peroxidation in its response to inhibitors as well as in its cofactor requirements. The stimulation of the reaction by a pyrophosphate group is in agreement with the observations of Hochstein et al. (9) on the microsomal lipid peroxidation system. They suggested that pyrophosphate might chelate the iron in some fashion, making it more active in catalyzing peroxidation, and observed, as we have done for the decarboxylation of retinoic acid (Fig. 2), that PP_i was active at low and less active at high concentrations. In addition, the pH maximum of 7.5 is the same as that of endogenous microsomal lipid peroxidation (8–10, 12). The data of Wills (11) appear to be in disagreement, but they refer to the oxidation of exogenous substrates and differ in other ways as well from data reported for endogenous lipids. Direct evidence for the mechanism cannot be obtained until the products and intermediates in this reaction are positively identified, but it seems a reasonable hypothesis that the decarboxylation is initiated by a free-radical mechanism resembling that in peroxidation. The decarboxylation step itself might be nonenzymatic since it occurred in the presence of heated microsomes in the ascorbate–Fe^{2+} system; the NADPH-linked portion of the mechanism is, however, enzymatic.

The indication that the product of the reaction might be an aldehyde also reflects the similarity of this reaction to lipid peroxidation. Tappel has found one product of linoleate peroxidation that reacts like an aldehyde (26); McCay and May have shown an aldehyde-containing product to result from peroxidation of arachidonic acid (27); and Süllmann very early demonstrated that carbonyl compounds resulted from oxidation of linolenic acid (28). The lower absorption maximum of the product indicates that the conjugated system has been shortened. The polar nature of the products, shown by silicic acid chromatography, indicates that the reaction yields products other than the simple C-14 aldehyde. These products possibly contain hydroxyl or hydroperoxide groups which have not led to chain scission.

The maximum of 50% decarboxylation achieved must reflect the random nature of the peroxidative attack or some other enzymatic destruction of retinoic acid. It does not reflect a nonenzymatic reaction, for this proceeds at too low a rate to account for 50% destruction in the short time in which the decarboxylation is completed.

Whether this particular microsomal decarboxylation is related to the previously reported in vivo and in vitro reactions (1–3) and what its physiological significance in retinoic acid metabolism might be remain to be demonstrated. Extensive peroxidation is usually restricted to in vitro systems, where the normal cellular integration has been destroyed. There is some evidence for in vivo formation of lipid peroxides in vitamin E deficiency (29), but reports regarding the ability of lipid peroxides to destroy enzymes (30–32), membranes (33), and vitamins (13) make it unlikely that this is a significant process in the healthy animal. However, without further evidence to the contrary, it cannot be ruled out that hydroperoxide formation could constitute an important pathway for the metabolism of retinoic acid.

Whether or not this oxidative decarboxylation is demonstrated to be significant in vivo, it is a very rapid reaction in vitro, and any further work on the metabolism of retinoic acid must take into account these peroxide-like reactions.

The authors thank Mrs. Yoko Tanaka for her excellent technical assistance.

This work was supported by Grant GB 3086 from the National Science Foundation and one of us, Anita Roberts, received support as a predoctoral fellow from Public Health Service Fellowship 5 Fl GM 30,014-01. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

Manuscript received 5 October 1967; accepted 26 March 1968.

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