have been reported recently. The role of a nuclear substituant on the Δ^4-steroid molecule for reduction of the ketone at carbon 3 to occur as discussed by Ringold *et al*(5), if it is in fact necessary, may have various possible *in vivo* counterparts in terms of protein-steroid interactions. The forward and reverse reactions involving the 3-hydroxyl-Δ^4 group merit further investigation to establish their significance in the biosynthetic or catabolic pathways of steroid metabolism.

ADDENDUM ADDED IN PROOF: The authors wish to point out an error in nomenclature in the article by Rosner, Horita and Forsham, *Endocrinology,* 1964, v75, 299. The compound called androstenediol should be designated as Δ^5 not Δ^{4-}. They demonstrate a conversion of the Δ^5-3β-hydroxyl group to the Δ^4-3 ketone. Our investigations are concerned with the allyl structure, Δ^4-3β-hydroxyl group.

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Alteration in Citrate Metabolism in Parathyroid Extract-Treated Calvaria.* (29678)

CHRISTYNA E. MECCA,† G. R. MARTIN, E. SCHIFFMANN AND P. GOLDHABER‡
(Introduced by I. Zipkin)
National Institute of Dental Research, Bethesda, Md. and Harvard School of Dental Medicine, Boston Mass,

Mouse calvaria, when grown in tissue culture under appropriate conditions, resorb when parathyroid extract (PTE)§ is added to the medium(1). During resorption, citrate levels are increased in the medium(2). Although bone contains large quantities of citrate relative to other tissues, the release of citrate bound to mineral can account for less than 1/40 of the total. The major proportion of the citrate is newly synthesized. Since citrate is a potent demineralizing agent and may be one of the acids concerned with mineral resorption, we have attempted to determine if the oxidative metabolism of citrate is altered during PTE-induced resorption.

Methods. In these studies the tissue culture methods developed by Goldhaber were employed(1,2). In general, calvaria weighing approximately 6 mg were obtained from 5-day-old mice and were attached to either a coverslip or a piece of 0.45 μ Millipore® fllter (HA) with a plasma clot. These preparations were placed in glass tubes containing 2 ml of a medium composed of 20% Gey's salt solution, 80% heated horse serum and 100 units/ml each of penicillin and streptomycin. PTE was added to the medium of

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‡ U.S.P.H.S. Research Career Development Awardee.

§ Lilly Parathormone.

the experimental cultures to give a final level of 0.5 units/ml. All tubes were gassed briefly with a mixture of 50% O_2 + 50% N_2, stoppered and placed horizontally in a rotating drum at 37°C. The media are changed and the tubes gassed every 2 days.

To measure the formation of $C^{14}O_2$ from the metabolism of various radioactive substrates by calvaria in culture, a narrow strip of filter paper impregnated with 0.5 ml of Hyamine was impaled on a stainless steel wire which was inserted into the bottom of a rubber stopper. The Hyamine-impregnated paper was inserted into the gas phase of the culture tube, and the tube stoppered. At various times the papers were removed from the wire, placed in a toluene-phosphor solution and counted in a Packard liquid scintillation counter. It was found that essentially all the $C^{14}O_2$ formed in the tube was present in the Hyamine-soaked filter paper.

To study $C^{14}O_2$ evolution by homogenates, calvaria were grown in culture with and without PTE. After 2 days the calvaria were removed from the tubes and ground in the cold in a loose glass homogenizer in Gey's salt solution containing 100 units/ml of both penicillin and streptomycin. In general, the homogenates contained 1 calvaria/ml. These homogenates were centrifuged at low speed to remove unbroken cells and debris. Two ml portions of this homogenate were placed in Warburg vessels containing a Hyamine soaked filter paper in the center well, various cofactors and radioactive substrates were added and the vessels were sealed and then shaken in a water bath at 37°C. After various times, the reaction was stopped by addition of 0.5 ml of 4 N HCl, and the Hyamine-soaked filter paper was assayed for radioactivity as previously described.

Calcium(3), phosphate(4) and citrate(5) analyses were conducted on the media from both control and experimental cultures. In general, the levels were similar to those reported previously(2). The values reported here are the averages of at least 3 determinations.

Results and discussion. To determine whether the increases in media citrate during resorption represented changes in the rate of citrate metabolism, we have studied the evolution of $C^{14}O_2$ from various radioactive substrates. In these studies, a C^{14}-labeled substrate was added to the fresh media given control and experimental calvaria on the second day in culture, and the $C^{14}O_2$ produced was measured on the fourth day. In the PTE-treated cultures, $C^{14}O_2$ production from citrate-1,5-C^{14} was found to be reduced, while $C^{14}O_2$ production from the other compounds appeared to be relatively unchanged (Table I). However, this does not necessarily mean that the metabolism of the other compounds via a different pathway or to a product other than CO_2 is not altered. In this regard Cohn(6) has reported reduced $C^{14}O_2$ evolution from citrate-1,5-C^{14}, succinate-1,4-C^{14} and fumarate-1,4-C^{14} and an increased $C^{14}O_2$ production from glucose-6-C^{14}, lactate-1-C^{14} and lactate-2-C^{14} by bone slices from PTE-treated animals.

TABLE I. Influence of PTE on Metabolism of Various Metabolites by Calvaria in Culture.*

Substrate	$C^{14}O_2$ (cpm) Control	PTE	Difference (%)
Citric acid-1,5-C^{14}	571 ±32	200 ±11	−65
Aspartic acid-4-C^{14}	548 ±31	488 ±29	−11
Glucose-U-C^{14}	229 ± 9	212 ± 8	− 7
Glutamic acid-5-C^{14}	920 ±35	866 ±40	− 6
Pyruvic acid-1-C^{14}	190 ± 9	188 ± 6	− 1
Pyruvic acid-2-C^{14}	153 ± 6	165 ±11	+ 8

* Substrates (approximately 50,000 cpm) were added to bones after second day in culture and $C^{14}O_2$ measured on 4th day.

In other studies, either citrate-1,5-C^{14} or isocitrate-5,6-C^{14} were added to the media in which control and experimental calvaria were incubated at start of the experiment and also after the calvaria had been in culture for 2 days. $C^{14}O_2$ production from citrate-1,5-C^{14} was not inhibited in the first 3 hours during which the calvaria were in culture with PTE but was reduced after 2 days (Table II). The production of $C^{14}O_2$ from isocitrate-5,6-C^{14} was not changed from normal by PTE-treatment at either time.

To rule out the possibility that the reduced oxidation of citrate-1,5-C^{14} by experimental cultures was due to an increased pool of citrate, we took advantage of the fact that there

TABLE II. Metabolism of Citrate-1,5-C^{14} and Isocitrate-5,6-C^{14} by Control and PTE-Treated Bone in Tissue Culture.*

Bone	Time in culture (days)	$C^{14}O_2$ (cpm) from Citrate-1,5-C^{14}	$C^{14}O_2$ (cpm) from Isocitrate-5,6-C^{14}
Control	0	154	32
PTE	0	140	44
Control	2	225	79
PTE	2	90	89

* Citrate-1,5-C^{14} (0.67 μc) or DL-isocitrate-5,6-C^{14} (0.67 μc) added to bones in tissue culture with or without PTE (0.5 units/ml) and $C^{14}O_2$ production measured after 3 hr.

TABLE III. Metabolism of Citrate-1,5-C^{14} and Isocitrate-5,6-C^{14} by Homogenates of Control and PTE-Treated Bone.*

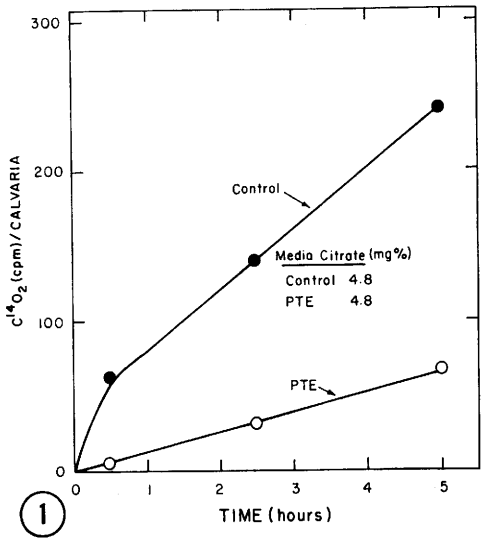
	Flask	NADP (μm/ml)	Homogenate Control	Homogenate PTE	$C^{14}O_2$ (cpm)
Citrate-1,5-C^{14}	1	3.2	0	0	42
	2	0	+	0	35
	3	3.2	+	0	415
	4	3.2	0	+	229
Isocitrate-5,6-C^{14}	5	0	+	0	21
	6	3.2	+	0	10,266
	7	3.2	0	+	9,544

* Total volume of reaction vessel was 3.2 ml. Two ml of homogenate (1 bone/ml) were added where indicated. Bones grown for 4 days in culture with or without 0.5 units PTE.

is a lag phase preceding citrate elevation in the media from PTE-treated calvaria on the second day in culture. Citrate-1,5-C^{14} was added to the media given control and experimental calvaria after the second day in culture and citrate levels and $C^{14}O_2$ production were measured (Fig. 1). In both cases $C^{14}O_2$ was produced at a linear rate. The rate of $C^{14}O_2$ production from citrate-1,5-C^{14} by experimental cultures was considerably less than that observed in the controls, whereas the media citrate levels were identical in the time periods studied.

The possibility that PTE altered the permeability of the cell to citrate was considered by studying the metabolism of citrate-1,5-C^{14} in homogenates prepared from calvaria. Experiments on homogenates prepared from control calvaria indicated that nicotinamide adenine dinucleotide phosphate (NADP) was a necessary cofactor for $C^{14}O_2$ production from citrate-1,5-C^{14} (Fig. 2). Nicotinamide adenine dinucleotide (NAD) could not substitute for NADP nor did it cause a further increase in $C^{14}O_2$ production in homogenates already fortified with NADP.

When citrate-1,5-C^{14} was added to homogenates prepared from control and experimental calvaria grown in culture for 2 days, it was found that less $C^{14}O_2$ was produced by an homogenate prepared from the experimental cultures than by that from the control (Table III). The magnitude of the inhibition is similar to that observed in intact



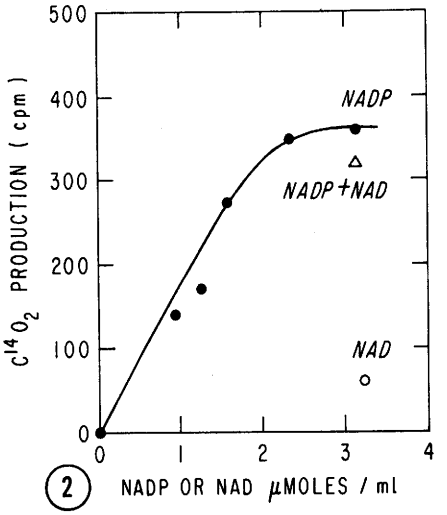


FIG. 1. Metabolism of citric acid-1,5-C^{14} by calvaria on the second day in culture.

FIG. 2. Citrate-1,5-C^{14} metabolism by bone homogenate.

calvaria. Considerably more $C^{14}O_2$ was produced from isocitrate-5,6-C^{14} than from citrate-1,5-C^{14} by the homogenates. There was no indication that the amount of $C^{14}O_2$ produced from isocitrate-5,6-C^{14} differed between the control and experimental group.

The generation of $C^{14}O_2$ from citrate-1,5-C^{14} is impaired in PTE-treated calvaria. This effect cannot be accounted for on the basis of an increased pool size or a change in permeability of the cell. The most likely possibilities are that the inhibition reflects decreased activities or levels of an enzyme or a cofactor. As reviewed by Krane *et al*(7) the generation of $C^{14}O_2$ from citrate-1,5-C^{14} first occurs when $C^{14}O_2$ is eliminated from α-ketoglutarate-1,5-C^{14} to form succinate. Aconitase, isocitric dehydrogenase and α-ketoglutaric dehydrogenase are involved in this series of reactions. We have not so far been able to measure the activities of the individual enzymes. However, since the production of $C^{14}O_2$ from isocitrate-5,6-C^{14} and glutamate-5-C^{14} is unchanged, it is unlikely that the activities of isocitric dehydrogenase or α-ketoglutaric dehydrogenase are affected. It is possible that citrate-1,5-C^{14} oxidation is impaired due to decreased levels of aconitase or that in the intact calvaria NADP levels are reduced. Either effect would tend to cause the accumulation of citrate observed after PTE-treatment. This may not be the only factor elevating citrate levels, since there is increased glucose utilization(2) and lactate(6) oxidation by PTE-treated bones indicating that the rate of citrate production may be increased.

Summary. We have studied the metabolism of several labeled compounds (as indicated by $C^{14}O_2$ production) by mouse calvaria grown in tissue culture with and without parathyroid extract (PTE). The generation of $C^{14}O_2$ from citrate-1,5-C^{14} is impaired in PTE-treated calvaria. This effect is evident in calvaria maintained 2 days in culture and does not appear to be due solely to an increased pool of citrate. A decreased production of $C^{14}O_2$ from citrate-1,5-C^{14} of similar magnitude is observed in homogenates prepared from PTE-treated calvaria. $C^{14}O_2$ production from isocitrate-5,6-C^{14} is not altered by PTE-treatment of the calvaria. This inhibition of citrate metabolism may account for the increase in citrate levels observed in PTE-treated calvaria.

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Photographic Elimination of Transients (PET): A Simple Technique for Retrieving Bioelectrical Signals from Noise.* (29679)

R. L. COLLINS (Introduced by L. C. Massopust Jr.)

Department of Neurochemistry, Cleveland Psychiatric Institute and School of Medicine, Western Reserve University, Cleveland, Ohio

Bioelectronic recording techniques have become a fundamental research tool in basic and applied neurophysiology and psychology. Although the refinement of electronic apparatus has enabled bioelectrical measurement and recording to be made with great precision, the presence of noise or random discharges from the biological preparation itself

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