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Influence of Endogenous Parathyroid Hormone on Citrate and Lactate Production by Bone *in vitro*.* (30105)

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The specific mechanism by which the parathyroid glands influence calcium homeostasis has not been established. Discovery of calcium-lowering factors from the parathyroids, thyroids, and adrenals(1,2,3) have made the problem even more complex. Clearly, however, the function of the classical parathyroid hormone is to maintain the circulating calcium level by altering kidney thresholds and mobilizing bone calcium. Suggestions for explaining the bone salt mobilization have included mitochondrial transport(4), alterations in RNA(5), and changes in bone cell carbohydrate metabolism(6,7,8). Various workers have reported apparently conflicting data on the effects of parathyroid extract on glucose breakdown by incubated bone tissue. Some have found that citrate production by the cells was altered(9), others have reported changes in lactate production(7,8), and still others have found no effect(10). In contrast to the above studies which were done primarily with the administration of parathyroid extract, the work reported here was carried out to determine whether or not changes in the level of *endogenous* parathyroid hormone secretion would have any effect on the production of citrate and lactate by bone tissue incubated *in vitro*.

Materials and methods. Male Holtzman rats, 180-200 g, were used throughout these studies. To alter production of endogenous parathyroid hormone, the animals were subjected to one or more of the following treatments: parathyroidectomy, nephrectomy, and/or peritoneal lavage. All surgery was performed under light ether anesthesia. Nephrectomy was done through a single, mid-ventral incision; parathyroidectomy was

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TABLE I. Control Values.

A. Plasma values at start of incubation		
1. Citrate	1.5 mg/100 ml	(0.060 mg total)
2. Lactate	10.0 mg/100 ml	(0.40 mg total)
3. Phosphate	10.0 mg/100 ml	
4. Calcium	10.0 mg/100 ml	
B. Sample calculation		
1. Dead bone—citrate		
a. Total citrate in media post-incubation		.177 mg
b. " " " " initially		.060 "
c. " released by dead bone (a-b)		.117 "
d. Dry wt of bone		.500 g
e. Citrate released—mg/g bone (c÷d)		.234 mg/g bone
2. Live bone—citrate		
a. Total citrate in media post-incubation		.652 mg
b. " " " " initially		.060 "
c. " " contributed by bone (a-b)		.592 "
d. Dry wt of bone		.500 g
e. Citrate contributed per gram of bone		1.184 mg/g bone
f. Citrate released—dead bone		.234 " "
g. Net citrate production (e-f)		.950 " "
3. Dead bone—lactate (calculated in same manner as citrate)		.300 " "

C. Whole femur citrate content—mg/g bone

Holtzman diet:	Non-incubated	Incubated
Controls	5.84 ± .78*	6.52 ± .56
Parathyroidectomized (4 hours)	5.50 ± .49	5.43 ± .78

* Mean $\pm \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

6 animals at each point.

done surgically using fine jeweler's forceps. The technique of peritoneal lavage, as well as the composition of the lavage fluids, has been described(11,12).

Following treatment of the animals, the femora were prepared and incubated as described earlier(13). The crudely-minced femora were placed in pooled rat serum (pH 7.4) under an atmosphere of 95% oxygen-5% carbon dioxide. A temperature of 37°C was maintained in a water bath. At the designated times the femora were removed from the media, and all reactions in the media were stopped by addition of sufficient 20% trichloroacetic acid for a final concentration of 5% trichloroacetic acid. Bones which had been subjected to repeated freezing and thawing were run simultaneously. These are referred to as dead bones, because cytological examination of the ultrastructure verified destruction of the cells.

Citrate was determined by the method of Saffran and Denstedt(14) and lactate, by the method of Barker and Summerson(15). For the incubations with radioactive glucose, one or two microcuries of uniformly-labelled D-glucose-C^{14} (28.5 mc/mM, Calbiochem) were added to the incubation media. The organic acids were separated chromatographically by the method of Ladd and Nossal (16); the spots containing the indicated acids were cut from the chromatogram and counted on a thin window gas flow counter.

The procedure used for decalcifying and defatting the bones for weighing was a modification of the method of Vaes and Nichols (17). This was done immediately following the incubation.

Results. Control values. The technique used in the incubation studies has been described elsewhere along with a discussion of calcium metabolism in this system(13). Given in Table I are typical plasma values for the various substances under consideration at the start of the incubation period. Also included is a sample calculation for de-

TABLE II. Net Media Citrate and Lactate Production by Femora—mg/g bone.

	Hours of incubation	
	2	6
Citrate		
Control (15)*	.95 ± .016	1.30 ± .096
Parathyroidectomized (9) (4 hr)	.86 ± .016†	1.06 ± .07†
Lactate		
Control (15)	13.8 ± 1.1	60.9 ± 3.8
Parathyroidectomized (9) (4 hr)	14.8 ± 1.3	46.8 ± 2.7†

* No. of animals.
† Differ from control $p < .005$.

termination of the amount of citrate produced and released into the media. Lactate is calculated in a similar manner. All citrate and lactate values are expressed as milligrams of the acid per gram of decalcified, fat-free bone.

The effect of incubation on the total citrate content of bones was determined by comparing values of bones that had been incubated to those of bones that had not been incubated. In no case was there any significant reduction in citrate content of the bones following incubation; however, it should be noted that with the comparatively large amount of citrate in bone that the standard errors of these values were of the same magnitude as the total amount released into the incubation medium.

Effect of parathyroidectomy on citrate and lactate production during incubation. For these experiments rats were parathyroidectomized 4 hours prior to removal of the femora for incubation. The effects on citrate and lactate production are summarized in Table II. Citrate levels in both groups remained relatively constant throughout the period studied indicating that citrate, while being released into the media, was also being metabolized. Lactate, on the other hand, accumulated throughout the incubation period. After 2 hours the lactate concentration was 15 times that of citrate; by 6 hours this had risen to a factor of fifty. Parathyroidectomy caused a decrease in citrate production which was detectable by the second hour of incubation. The effect on lactate production could not be detected unless the incubation was carried 6 hours. It must be pointed out that due to the relative accumulation of these 2 acids in the media, the standard error of the lactate values is greater than the total citrate released.

Incorporation of C^{14}-glucose into media citrate and lactate. Uniformly labelled C^{14}-glucose was added to the media prior to incubation with femora from control and parathyroidectomized rats. The incorporation of label into citrate and lactate is summarized in Table III. All values are given as counts per minute per gram of bone. Radioactivity recovered in citrate was greater at 2 hours, decreasing approximately 50% by 6 hours. Incorporation of the label into lactate was slight at 2 hours (approximately ten per cent of the amount that appears in the citrate at the same time). The amount of tagged lactate increased over the 6-hour incubation. Parathyroidectomy produced a decrease in incorporation of the label into citrate; this was readily detectable by 2 hours of incubation. In a manner similar to the effect on total lactate, parathyroidectomy could be shown to affect the incorporation of label only if the incubation were extended to 6 hours.

To prove viability of the bone fragments over the course of the incubation, radioactive glucose was added to the media after 4 hours of incubation, and the incubation was continued for 2 additional hours. Radioactivity

TABLE III. Incorporation of C^{14}-Glucose into Media Citrate and Lactate—Whole Femur c/m/g bone.

	Hr of incubation	
	2	6
Citrate		
Control	1000 ± 100*	543 ± 9
Parathyroidectomized	601 ± 91†	364 ± 33†
Controls incubated 4 hr; C^{14}-glucose then added to media; incubated 2 more hr	896 ± 68	
Lactate		
Control	83 ± 3	837 ± 75
Parathyroidectomized 6 animals at each point.	73 ± 19	567 ± 85†

* Control citrate value for 2-hour incubation set at 1000 for purposes of comparison, and all other radioactivity values adjusted proportionally.
† Differ from control $p < .005$.

TABLE IV. Comparison Between Diaphysis and Metaphysis.

	Hr of incubation			
	2		6	
	Metaphysis	Diaphysis	Metaphysis	Diaphysis
A. Net media organic acid production—mg/g bone				
Citrate				
Control	1.19 ± .15	2.62 ± .21	1.87 ± .21	2.75 ± .05
Parathyroidectomized	1.06 ± .18	1.53 ± .12†	2.23 ± .05	2.37 ± .07†
Lactate				
Control	16.8 ± 2.9	15.0 ± 1.5	129.0 ± 11.9	87.3 ± 8.0
Parathyroidectomized	14.2 ± 2.3	15.3 ± 4.5	67.7 ± 7.1†	63.4 ± 5.9†
B. Incorporation of C^{14}-glucose into citrate and lactate—c/m/g bone				
Citrate				
Control	1000 ± 82*	2671 ± 435	885 ± 105	3385 ± 290
Parathyroidectomized	803 ± 274	1146 ± 221†	1311 ± 222	1782 ± 248†
Lactate				
Control	103 ± 6	92 ± 6	291 ± 12	192 ± 20
Parathyroidectomized	86 ± 2†	84 ± 5	188 ± 18†	184 ± 25

8 animals at each point.
* Control citrate value for 2-hour metaphysis incubation set at 1000 for purposes of comparison, and all other radioactivity values adjusted proportionally.
† Differ from control $p < .005$.

incorporation into citrate was in very good agreement with the incorporation found for the initial 2 hours of a companion experiment, demonstrating the viability of the cells through 6 hours of incubation.

Organic acid production by metaphysis and diaphysis. For these experiments the metaphysis and diaphysis, prepared as reported earlier(13), were incubated separately. Comparative data for the metaphysis (trabecular bone) and the diaphysis (compact bone) are given in Table IV. The following differences should be noted: 1. Citrate production is greater in the diaphysis; only here does parathyroidectomy significantly decrease the rate of its production. 2. Lactate production tends to be higher in the metaphysis; the effects of parathyroidectomy are demonstrable in both areas but only after 6 hours of incubation. 3. The incorporation of radioactivity follows in every case the differences demonstrated for the stable acids.

Effect of endogenous parathyroid hormone levels on citrate production. The last group of experiments compares citrate production after 2 hours of incubation of whole femora from animals with differing levels of endogenous parathyroid secretion. The results, summarized in Table V, are listed in order of decreasing hormone secretion in the animal from which the femora were removed. Four hours of peritoneal lavage with NaF-containing lavage fluid resulted in a 25% increase while after 12 hours of parathyroidectomy a 20% reduction was observed. (For rationale of NaF lavage, see 12.)

Discussion. Bone cells incubated *in vitro* in serum will continue to metabolize and produce measurable amounts of citrate and lactate. Under the conditions described in this study, the major end product accumulated is lactate. This is in agreement with the work of others who have used synthetic media(7, 8). Lactic acid appears to accumulate over

TABLE V. Effect of Time of Parathyroidectomy and of Parathyroid Stimulation on Citrate Production—mg/g bone.

Treatment	2 hours of incubation
Parathyroid-intact (4)* NaF lavage (4 hr)	1.168 ± .023†
Parathyroid-intact (12) lavage (6 hr)	1.088 ± .037†
Parathyroid-intact (4) lavage (4 hr)	1.000 ± .011†
Nephrectomized (24 hr) (12)	1.002 ± .011†
Parathyroid-intact controls (15)	.949 ± .016
Parathyroidectomized 2 hr (4)	.910 ± .014
Parathyroid-intact lavage 4 hr (12 mg/100 ml Ca in lavage fluid)	.879 ± .061
Parathyroidectomized 4 hr (9)	.861 ± .016†
Parathyroidectomized 12 hr (6)	.793 ± .043†

* No. of animals.
† Differ from control $p < .005$.

the entire 6-hour period. Citrate, on the other hand, builds up early and remains relatively constant throughout the incubation period. Since dead bone values are subtracted from the total, production and reutilization of citrate by bone cells are indicated.

Studies with radioactive glucose indicate that incorporation into citrate appears much earlier than incorporation into lactate, despite the fact that more total lactate is being produced. Since pyruvate exists as a common intermediate in the breakdown of glucose to either citrate or lactate, the preferential, early incorporation into citrate by the bone cells *in vitro* presents somewhat of a problem. Several possible explanations may be offered: 1. Two distinct pathways, glucose to citrate and glucose to lactate, exist which do not have a common intermediate. In the light of present knowledge of biochemical pathways, such a suggestion is considered untenable. 2. Intracellular compartmentalization exists which allows citrate and lactate production to take place at different rates within the same cell. While this cannot be ruled out as a possibility, its verification must await a better understanding of the relationships of sub-cellular structure and function. 3. Two different populations of bone cells exist which differ in regard to the end product of glucose metabolism and possibly the rate at which they utilize glucose. Radioglucose studies with the metaphysis and diaphysis incubated separately indicate that the two populations (that is, the cells which produce mainly citrate and the cells which produce mainly lactate) are found throughout bone but that the ratio of these 2 populations may differ in the two areas of bone. This third explanation appears the most attractive and is preferred.

If one accepts the premise that there exist 2 populations of bone cells in regard to their methods of glycolysis, several additional observations may be made: 1. Studies with the radioactive glucose indicate that either the breakdown of glucose through citrate proceeds at a much faster rate or that those cells producing lactate have an endogenous supply of glycogen which is used preferentially. 2. Parathyroid hormone normally affects both types of cells, but its effect on the citrate-producing cells can be detected earlier.

These studies may also add additional information in regard to parathyroid action on bone cells. Since both citrate and lactate production are affected by the absence of parathyroid hormone and if the hormone has only *one* fundamental action, it might be to alter the rate of a reaction prior to formation of pyruvate, a common intermediate in the glycolytic pathway. These studies are of little help in pinpointing the site of such a reaction, whether it be in the Emden-Meyerhof pathway or the pentose shunt. The pentose shunt has been implicated by the work of Hekkelman(18). Also, these studies do not negate the possible direct effects of parathyroid hormone on the tricarboxylic acid cycle itself. The second point in regard to parathyroid action is that changes in endogenous levels of the hormone are very rapidly reflected in this system. Twenty or twenty-five per cent changes can be seen 4 hours after parathyroidectomy or stimulation of additional endogenous production. Preliminary studies at 2 hours indicate that changes can be detected within this time period, but the significance of these changes has not been established.

A final point of emphasis is the difference between the two parts of the femur studied separately. The effects of parathyroid hormone on citrate production are seen primarily in the diaphysis. This section consists mainly of compact bone; the most numerous cell type is the osteocyte although osteoblasts and osteoclasts are also present(19). The parathyroid effect on lactate production was seen in both sections but appeared greater in the metaphysis. This section consists mainly of spongy bone; the cell population consists of a mixture of mesenchyme cells, osteoblasts, osteoclasts, and relatively young osteocytes. The metaphysis is a site where major remodelling of bone occurs. Processes in bone which involve calcium removal are bone remodelling (salt removal in bone reshaping) and bone resorption (salt removal for calcium homeostasis)(20). While the data here and

elsewhere(13,20) are not yet conclusive, one might speculate that these are two distinct processes which may be present in different ratios in different areas of bone. It has been suggested that the homeostatic process is more active in the diaphysis, while remodelling takes place primarily in the metaphysis (13).

Summary. Femora removed from rats with varying levels of endogenous parathyroid secretion were incubated in pooled rat serum. Samples of the media were analyzed for citrate, lactate, and C^{14}-incorporation into these 2 acids from labelled glucose added to the incubation media. Both citrate and lactate production by bone cells decreased within 4 hours after parathyroidectomy. Differences in citrate production were readily detectable in a 2-hour incubation; 6 hours of incubation were required to show differences in lactate formation. While lactate accumulated in the media throughout the incubation, citrate reached a peak and leveled off suggesting recycling. The largest incorporation of radioactivity from glucose into citrate was also seen at 2 hours. At this time incorporation into lactate was negligible; however, by 6 hours radioactivity in lactate equalled the highest value observed for citrate. Incubation of diaphyseal and metaphyseal separately demonstrated greater citrate production in the diaphysis; conversely, the metaphysis was the site of greater lactate production. Data from the studies with radioactive glucose confirm all of the findings for total organic acid production. These data are interpreted as suggesting that there are 2 populations of bone cells which differ as to their primary end product of glucose metabolism. If parathyroid hormone has only one site of action on both these cell populations, it would seem to affect the rate of a reaction prior to the formation of pyruvate in the glycolytic pathways.

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