ylation is a major mechanism if it is not the only one.

The formation of VII also could have been brought about directly by the irradiation of 1,3-dimethyluracil. During this process, the steps leading to the formation of VII occurred only after almost complete conversion of the starting material to I (7 hr.); VII was isolated and identified.

The irradiation on N,N'-dimethylmalonamide (VII) did not bring appreciable change even after 4–5 days at a 10^{-2} concentration.

The mechanism of this irradiation process seemed to be a free radical reaction. The rate of the photo-oxidation reaction at 10^{-3}–10^{-4} M appeared to be a function of its concentration; however, at higher concentrations the rate seemed independent of the concentration of I, about the same reaction time being observed for 3×10^{-3} M (21–25 hr.), 6×10^{-3} M (21 hr.) and 1.2×10^{-2} M (22–28 hr.) with the same reaction conditions. The kinetics of decarbonylation (II to VII) were pseudo zero order at 10^{-4} M as shown by a plot of optical density *vs.* time.

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Concerning the Mechanism of Action of Parathyroid Hormone. II. Metabolic Effects[1,2]

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The effects of varying parathyroid status on arteriovenous differences of pyruvate, lactate and citrate were studied in several tissues in the dog. Bone was shown to be an important source of circulating citrate and this citrate production by bone was dependent upon parathyroid hormone levels. Citrate uptake was shown only by the kidney, but a hormonal effect on this process, if it occurs, was not clearly demonstrated. Interesting trends suggesting a hormonally stimulated utilization of pyruvate and production of lactate and citrate were observed in various tissues in addition to bone and kidney. These findings are discussed in terms of the mechanism of action of parathyroid hormone.

In recent years, attention has focused upon the importance of citric acid in bone metabolism, yet the true significance of citrate in bone has remained obscure.[3] Parathyroid hormone influences citric acid metabolism[4,5] and, recently, conclusive evidence that this influence is a direct effect of the hormone was provided by the experiments of Freeman and Chang[6] and Elliott and Freeman,[7] who demonstrated that the rise in the level of citric acid in the blood that accompanies nephrectomy is abolished by a prior parathyroidectomy.

In previous publications of the present series,[8,9] this relationship between parathyroid status and citrate metabolism has been confirmed and extended. It was shown that, following the intravenous injection of parathyroid hormone in dogs, a sharp rise in the level of blood citrate occurred reaching a peak one hour following the injection. The rise in citrate was followed closely by a parallel rise in inorganic phosphate, while the calcium level did not increase until 4–6 hours following the injection. The release of citrate by bone and its dependence upon parathyroid hormone, was indicated. Data demonstrating that citrate can exchange for phosphate on the surface of the bone crystals, with a resulting increase in the solubility of the mineral phase, also was presented.

In the present investigations, the uptake and release of pyruvate, lactate and citrate by various tissues, in addition to bone, and the influence of the parathyroid hormone upon such metabolic activity, has been investigated by means of the technique of arteriovenous differences. These studies were carried out in thyroparathyroidectomized, normal and parathyroid-injected dogs.

Methods

Mongrel dogs were thyroparathyroidectomized and used two days later for blood collection. These animals received 600,000 units of procaine penicillin intramuscularly following the operation. The parathyroid treated animals received one or two subcutaneous injections of parathyroid extract[10] at various intervals (5 to 24 hours) prior to blood collection, at a level of 100 units per kilo. This amount of hormone was selected as one which would elicit a major response in each animal. Effects of a similar nature undoubtedly could be obtained at a lower dosage.[11,12] Three of the control animals were sham operated.

Mixed bone blood was collected from a catheter inserted in a small hole drilled in the spongiosa of the femur of the dog, a technique described earlier.[8] As before, Sr^{89} was given five minutes before blood collection began to permit a comparison of Sr^{89} levels in the catheter blood with arterial levels. Since, in the early time periods, the Sr^{89} is effectively "cleared" in passing through bone and only bone,[9] this provided a rough measure of the proportion of the catheter sample which was truly venous outflow from bone. In many instances, several consecutive samples of catheter blood were obtained to afford a better estimate of venous outflow from bone. A corresponding arterial blood sample was drawn at the midpoint of each bone blood collection, for calculations of A-V differences. In addition to that from

(1) This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project, Rochester, New York.

(2) Taken in part from a thesis submitted by H. E. Firschein in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry, University of Rochester, 1958.

(3) T. F. Dixon and H. R. Perkins, "The Biochemistry and Physiology of Bone," G. H. Bourne, ed., Academic Press, Inc., New York, N. Y., 1956, Chap. 11.

(4) F. Dickens, *Biochem. J.*, **35**, 1011 (1941).

(5) N. Alwall, *Acta Med. Scand.*, **116**, 337 (1944).

(6) S. Freeman and T. S. Chang, *Am. J. Physiol.*, **160**, 341 (1950).

(7) J. R. Elliott and S. Freeman, *Endocrinology*, **59**, 181 (1956).

(8) W. F. Neuman, H. Firschein, P. S. Chen, Jr., B. J. Mulryan and V. DiStefano, THIS JOURNAL, **78**, 3803 (1956).

(9) H. Firschein, G. Martin, B. J. Mulryan, B. Strates and W. F. Neuman, *ibid.*, **80**, 1619 (1958).

(10) Purchased from the Eli Lilly Company, Indianapolis, Ind.

(11) L. C. Miller, *J. Am. Pharm. Assoc.*, **27**, 90 (1938).

(12) C. I. Bliss and C. L. Rose, *Am. J. Hyg.*, **31**, 79 (1940).

bone, blood was also obtained from the carotid artery, jugular vein, femoral vein, renal vein and mesenteric vein.

All animals were anesthetized with Dial (diallylbarbituric acid) prior to blood collection. Heparin was administered as required, in order to prevent the blood from clotting in the bone catheter. The test-tubes used for blood collection also contained small quantities of heparin. All analyses reported are for plasma.

Calcium was determined according to the method of Toribara, Dewey and Warner.[13] Citrate was converted to pentaboromoacetone and determined colorimetrically.[14] Lactate was determined by the method of Barker and Summerson.[15] Pyruvate was determined colorimetrically as the 2,4-dinitrophenylhydrazone, according to the method of Friedemann and Haugen,[16] as modified by Markees[17] and Lichtenbelt and Florijn.[18]

In order to determine whether the crude parathyroid hormone preparation used in these experiments was contaminated with thyroid hormone, iodine analyses (courtesy W. B. Mason) were performed on portions of the parathyroid extract obtained from the Eli Lilly Company. These extracts were essentially iodine-free, indicating the absence of the thyroid hormone.

Results and Discussion

Arteriovenous differences (venous minus arterial) in citrate, pyruvate and lactate with additional pertinent data have been assembled in Tables I and II and Fig. 1. A positive figure indicates

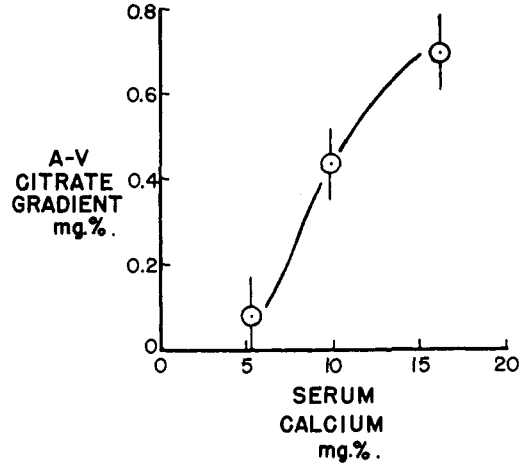

Fig. 1.—The relation between parathyroid status as indicated by the serum calcium level and production of citrate by bone as indicated by arteriovenous differences observed in bone catheter-collected blood. Perpendicular lines represent the standard error of the means.

that the tissue or area released the metabolite into the blood, while a negative difference indicates metabolite uptake.

The study of arteriovenous differences can be a powerful tool in determining net biochemical events *in vivo*. In these investigations, however, certain limitations are readily apparent. Venous samples, except from the renal and mesenteric veins, represent samples of blood from areas containing more than one tissue. Moreover, the levels of pyruvate and lactate in plasma are somewhat variable and

(13) T. Y. Toribara, P. A. Dewey and H. Warner, *Anal. Chem.*, **29**, 540 (1957).

(14) R. H. Ettinger, L. R. Goldbaum and L. H. Smith, Jr., *J. Biol. Chem.*, **199**, 531 (1952).

(15) S. B. Barker and W. H. Summerson, *ibid.*, **138**, 535 (1941).

(16) T. E. Friedemann and G. E. Haugen, *ibid.*, **147**, 415 (1943)

(17) S. Markees, *Experientia*, **7**, 314 (1951).

(18) H. Van M. Lichtenbelt and E. Florijn, *Biochim. et Biophys. Acta*, **8**, 349 (1952).

TABLE I

AVERAGED ARTERIAL LEVELS[a]

Substance	Parathyroid-ectomized	Control	Parathyroid injected
Calcium	5.3 ± 1.1 (4)	9.8 ± 0.8 (8)	16.1 ± 0.7 (9)
Citrate	2.8 ± 0.3 (4)	2.8 ± 0.3 (8)	4.4 ± 0.6 (9)
Lactate	26 ± 9.8 (4)	18 ± 1.9 (8)	30 ± 3.6 (6)
Pyruvate	2.1 ± 0.7 (4)	1.8 ± 0.3 (8)	2.5 ± 0.2 (6)

[a] All values expressed in mg. per 100 ml. of serum plus or minus one standard error of the mean. Numbers in parentheses indicate the number of animals studied.

are influenced by many physiological stresses other than the one under study, *i.e.*, parathyroid status. Because of these problems, only a few metabolic changes could with certainty be correlated with the parathyroid hormone level. Most of these statistically significant data were concerned with the metabolism of citrate.

Significant production of citrate was observed only in the blood collected from the bone catheter. Furthermore, these arteriovenous differences varied directly with the parathyroid status. In the parathyroidectomized animals the V-A difference was not significantly different from zero. In the control animals the average V-A difference was significantly positive, 0.44 mg. %, and in the hormone-injected animals, the value was significantly greater, 0.70 mg. %. If these V-A differences are corrected by Sr^{89} clearance values (25, 15, 21%) and arterial levels, the averaged citrate contents of venous blood *from bone* are approximately 3.2, 5.4 and 8.2 mg. % in the three groups, respectively. There is reason to question whether this extra citrate was derived from cells in bone or in marrow or in both. While proof is lacking, several pieces of evidence suggest that bone, not marrow, was the source of the released citrate. First, in an earlier study, a hole drilled directly into the marrow cavity released blood containing a smaller citrate gradient than a corresponding hole drilled into the spongiosa.[8] Furthermore, in several instances when the blood samples failed to show a Sr^{89} clearance no citrate gradients were observed. This correlation between the removal of radiostrontium, attributable only to bone, and the production of citrate, coupled with the correlation between citrate gradients and parathyroid status, is strongly suggestive that bone is an important, if not the primary, source of circulating citrate.

Significant uptake of citrate was shown only by the kidney. Here, the arteriovenous gradient seemed to be related to the arterial level of citrate and, perhaps, also to the parathyroid status. The average percentages removed by the kidney were 24, 27 and 34 in the parathyroidectomized, control and injected animals, respectively. Herndon and Freeman[19] and Herrin and Lardinois[20] have recently stressed that only the arterial level of citrate controls its oxidation rate in the kidney. However,

(19) R. F. Herndon and S. Freeman, *Am. J. Physiol.*, **192**, 369 (1958).

(20) R. C. Herrin and C. C. Lardinois, *Proc. Soc. Expti. Biol. Med.*, **97**, 194 (1958).

TABLE II

ARTERIOVENOUS DIFFERENCES AS AFFECTED BY PARATHYROID STATUS[a]

Substance	Source	No. of animals	Venous arterial gradient A Parathyroid-ectomized	B Control	C Parathyroid injected
Citrate	1—Renal vein	3,3,3	−0.7 ± 0.2	−0.6 ± 0.2	−1.4 ± 0.2
	2—Bone catheter	4,8,9	.1 ± .1	.4 ± .1	0.7 ± .1
	3—Mesenteric vein	4,5,3	− .2 ± .3	.2 ± .2	.3 ± .3
	4—Femoral vein	4,5,3	− .1 ± .3	.2 ± .3	.2 ± .3
	5—Jugular vein	4,5,3	− .2 ± .1	.3 ± .2	.3 ± .1
Lactate	6—Renal vein	3,3,3	−3.2 ± 1.9	−1.7 ± 1.9	.5 ± 1.9
	7—Bone catheter	4,8,7	−1.3 ± 2.2	0.2 ± 1.5	2.3 ± 1.7
	8—Mesenteric vein	4,5,3	−0.5 ± 3.1	.7 ± 2.8	−0.9 ± 3.5
	9—Femoral vein	4,5,3	−2.8 ± 3.6	.9 ± 3.2	9.8 ± 4.1
	10—Jugular vein	4,5,3	0.1 ± 3.0	1.3 ± 3.0	4.4 ± 3.5
Pyruvate	11—Renal vein	3,3,3	.0 ± 0.2	−0.2 ± 0.2	−0.4 ± 0.2
	12—Bone catheter	4,8,6	.0 ± .2	− .5 ± .1	−0.5 ± .1
	13—Mesenteric vein	4,5,3	.0 ± .3	− .5 ± .2	−1.1 ± .3
	14—Femoral vein	4,5,3	.3 ± .2	− .3 ± .2	−0.2 ± .3
	15—Jugular vein	4,5,3	.2 ± .3	.1 ± .1	0.0 ± .3

[a] The following comparisons were significant ($p < 0.05$)

A1 *vs.* C1	A5 *vs.* C5	A1 *vs.* 0
B1 *vs.* C1	B5 *vs.* C5	B1 *vs.* 0
		C1 *vs.* 0
A2 *vs.* B2	A9 *vs.* C9	
A2 *vs.* C2		B2 *vs.* 0
B2 *vs.* C2	A13 *vs.* C13	C2 *vs.* 0
A3 *vs.* B3 + C3	A14 *vs.* B14 + C14	

the question of whether parathyroid hormone also has an effect on renal oxidation of citrate cannot be regarded as unequivocally settled.

The present data and those published by Martensson[21] give the impression that bone and kidney are the principal organs concerned with the formation and oxidation of circulating citrate. Martensson reported positive gradients of citrate in the femoral vein. In a footnote he stressed that the citrate could have arisen from muscle *or* bone. Since in the present experiments the femoral vein showed smaller gradients than did the blood obtained from bone, muscle does not appear to be a major contributor of serum citrate.

Besides the kidney and bone, intestine and liver may also participate in citrate metabolism. There was some indication that, under parathyroid stimulus, the intestine released citrate into the mesenteric vein and both Martensson[21] and Harrison and Harrison[22] have reported positive arteriovenous differences in the portal circulation. Martensson also deduced that the liver has a limited ability to oxidize citrate. Unpublished results on the isolated, perfused liver, though fragmentary, indicated no appreciable oxidation at high levels of citrate in the perfusate.[23]

Interesting trends also were observed in the arteriovenous differences of both pyruvate and lactate. In general, pyruvate utilization and lactate production seemed to increase with increasing levels of parathyroid hormone. These trends were not limited to bone and kidney suggesting a generalized cellular effect of the hormone. Unfortunately, few of these changes were statistically significant. It is of interest, however, that the arteriovenous differences in pyruvate and lactate were shifting in opposite directions with changes in parathyroid status. The reported fall in the level of blood glucose after administration of parathyroid hormone[24-28] raises the possibility that the utilization of glucose, like pyruvate, for citrate production may be stimulated by the hormone.

It is too early to speculate on the means by which parathyroid hormone affects citrate production. The earlier report[8] of an interaction between Coenzyme II and crude extracts of the hormone has been confirmed, but there was evidence that the agent affecting Coenzyme II was separable from the calcium-mobilizing principle. Final evaluation requires the use of more purified components.[29] Apart from this problem, certain broad generalizations can already be made. Since the citrate content *in* bone[4] as well as citrate release *from* bone varies directly with parathyroid status, the hormone must cause increased production of citrate by bone cells. Certainly, present evidence renders unlikely the release of citrate stores or the decreased utilization of circulating citrate as plausible explanations of observed events. Increased citrate production could result from either a stimulated conversion from precursors or an inhibited utilization of citrate itself. Since bone already has been shown to contain little or no isocitric dehydrogenase,[30] the most likely effect of the hormone, at the moment, seems to be a stimulation of the conversion of the carbohydrate metabolites (pyruvate or glu-

(21) J. Martensson, *Acta physiol. Scand.*, **1**, Suppl. 2 (1940–1941).

(22) H. C. Harrison and H. E. Harrison, *Federation Proc.*, Abstr., **17**, 66 (1958).

(23) D. E. Haft and L. L. Miller, private communication.

(24) S. Seelig, *Z. ges. exptl. Med.*, **78**, 796 (1931).

(25) C. I. Parhon and H. Derevici, *Compt. rend. soc. biol.*, **107**, 388 (1931).

(26) J. Olmer, J. Paillas and B. Sicnasi, *ibid.*, **121**, 78 (1936).

(27) J. Olmer and J. E. Paillas, *Presse med.*, **44**, 1418 (1936).

(28) A. Y. Rappeport, *Problemes endocrinol. (U.S.S.R.)*, **8**, 3 (1938); *C. A.*, **34**, 8019 (1940).

(29) H. Rasmussen, *J. Biol. Chem.*, **229**, 781 (1957).

(30) T. F. Dixon and H. R. Perkins, *Biochem. J.*, **52**, 260 (1952).

cose?) to citrate. Such conversions should cause a lowering of the *p*H in the vicinity of the affected bone cells, an observation reported by Cretin[31] some years ago.

It has been known[32] that the circulating fluids are supersaturated with respect to bone mineral and that some such localized production of acid or surface-acting ion such as citrate or carbonate is needed to account for the normal levels of circulating calcium and phosphate.[9,32] It is, perhaps, surprising, however, that the effectiveness of citrate in solubilizing bone mineral is not ascribable to its chelation of calcium ion.[9]

Thus, it is not clear how the hormone increases citric (and others?) acid production but it is clear how variations in acid production in bone regulates the solubility equilibria involving bone mineral and the extra cellular fluids.

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ROCHESTER, NEW YORK

(31) A. Cretin, *Presse med.*, **59**, 1240 (1951).

(32) W. F. Neuman and M. W. Neuman, "The Chemical Dynamics of Bone Mineral," University of Chicago Press, Chicago, Ill., 1958.

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

Carbodiimides. VIII.[1] Observations on the Reactions of Carbodiimides with Acids and Some New Applications in the Synthesis of Phosphoric Acid Esters

BY MICHAEL SMITH, J. G. MOFFATT AND H. G. KHORANA

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The reactions of a variety of acids (in particular, mono- and diesters of phosphoric acid) with a group of carbodiimides in the presence of tertiary bases have been studied. The carbodiimides chosen were dicyclohexyl-, di-*p*-tolyl- and di-*t*-butylcarbodiimides and the bases used were pyridine and tri-*n*-butylamine. The mechanism of reactions of carbodiimides with acids is discussed in the light of these findings. The formation of diesters of phosphoric acid by the reaction of a monoalkyl phosphate with dicyclohexylcarbodiimide in the presence of an alcohol has been examined and it has been demonstrated that this synthesis can occur by two discrete mechanisms. The present studies have resulted in new and improved procedures for the synthesis of ribonucleoside-2′,3′ cyclic phosphates, diesters of pyrophosphoric acid, nucleoside-5′ monoalkyl phosphates and simple diesters of phosphoric acid.

Carbodiimides have proved to be valuable synthetic reagents and, by their use, new methods for the synthesis of esters of ortho- and pyrophosphoric acids,[1,2] nucleotide coenzymes[3] and related compounds,[4] cyclic phosphates,[5] polynucleotides,[6] nucleoside-5′ phosphoramidates[7] and peptides[8] have been developed. These applications all involve the reaction of acids with carbodiimides. In general, reaction occurs rapidly with a variety of acids[9] and, in the absence of competitive reactants such as amino compounds, leads to the formation of anhydrides. Carboxylic acids can, however, also yield the corresponding N-acylureas. The substituents on the carbodiimide molecule, the acid and the solvent have been found to influence the course of these reactions.[9] While general schemes for the mechanism of carbodiimide reactions have been suggested,[9] observations made recently in this Laboratory on the effects of tertiary bases on the reactions of phosphate esters with carbodiimides[3d] stimulated us to examine somewhat more closely the factors influencing these reactions. This study, in turn, has led to further synthetic applications in the phosphate field. In the present communication, the results of experiments performed primarily to throw light on the reaction mechanisms are presented and discussed first while the synthetic applications are described at the end.

Results

In the present work, dicyclohexyl-, di-*p*-tolyl- and di-*t*-butylcarbodiimides have been studied. Dicyclohexylcarbodiimide has been used in most of the synthetic work involving carbodiimides and is therefore regarded as a standard. Di-*p*-tolylcarbodiimide was chosen as a representative of carbodiimides containing electron-withdrawing substituents, while the reactions of di-*t*-butylcarbodiimide might give an indication of steric effects in the reactions of carbodiimides. Pyridine and tri-*n*-butylamine were chosen as organic bases of widely differing strengths (pK_a's in water, 5.19 and 10.89,[10] respectively). Reactions of mono- and diesters of phosphoric acid and of acetic acid were studied and

(1) Paper VII, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3741 (1957).

(2) (a) H. G. Khorana, *Can. J. Chem.*, **32**, 227 (1954); (b) H. G. Khorana and A. R. Todd, *J. Chem. Soc.*, 2257 (1953).

(3) See for example (a) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954); (b) E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956); (c) N. A. Hughes, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 3733 (1957); (d) M. Smith and H. G. Khorana, THIS JOURNAL, **80**, 1141 (1958).

(4) These include mixed anhydrides derived from nucleoside-5′ phosphates and (a) sulfuric acid, P. Reichard and N. R. Ringertz, THIS JOURNAL, **79**, 2025 (1957); (b) carboxylic acids, P. T. Talbert and F. M. Huennekens, *ibid.*, **78**, 4671 (1956); (c) amino acids, P. Berg, *Federation Proc.*, **16**, 152 (1957).

(5) (a) C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954); (b) G. M. Tener and H. G. Khorana, *ibid.*, **77**, 5349 (1955); (c) H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, *ibid.*, **79**, 430 (1957); (d) T. Ukita, K. Nagasawa and M. Irie, *Pharm. Bull. (Tokyo)*, **5**, 121 (1957).

(6) (a) H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, THIS JOURNAL, **79**, 1002 (1957); (b) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958); (c) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *ibid.*, **80**, 6223 (1958).

(7) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 4240 (1957); R. W. Chambers and J. G. Moffatt, *ibid.*, **80**, 3752 (1958).

(8) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955); H. G. Khorana, *Chemistry & Industry*, 1087 (1955).

(9) H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(10) P. Damsgaard-Sorensen and A. Unmack, *Z. physik. Chem.*, **A172**, 389 (1935).