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Metabolic Studies of Human Bone *In Vitro*. II. Changes in Hyperparathyroidism *

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Generalized bone disease has long been recognized as a feature of hyperparathyroidism, but the functional disturbance at the tissue level responsible for its appearance has not been studied extensively. The demonstration that human bone metabolism may be examined directly by incubation of fresh biopsy samples *in vitro* and the establishment of normal rates for certain metabolic functions in normal adult human bone (1) opened the way to re-examination of this problem.

The purpose of this paper is to report the results obtained in bone removed at biopsy from a group of eight surgically proven hyperparathyroid patients.

These suggest the existence of two biochemically distinct skeletal metabolic responses to hyperparathyroidism, a view that appears to be supported by prior animal studies (2–6), and the results of some supplementary experiments using the rat as a model.

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Methods

Bone samples from eight hyperparathyroid patients ranging in age from 32 to 78 years, two males and six females, were used. Four had adenomata proven at operation, two had parathyroid hyperplasia, and one had a parathyroid carcinoma. All were cured of hyperparathyroidism by extirpation of the appropriate parathyroid tissue. Samples of mixed bone (cortical and trabecular) were removed from the iliac crests of these patients by open biopsy for metabolic study either before or at the time of parathyroid exploration. The remaining patient exhibited renal failure, raised serum calcium and alkaline phosphatase levels, and a pathologic fracture of the patella associated with cystic change. After patellectomy, half of the bone was submitted for histologic examination and the remainder used for metabolic studies. This patient died suddenly in renal failure, and permission for post-mortem examination of the parathyroids was not obtained. A summary of the pertinent findings in these patients is presented in Table I.

Daily total urinary hydroxyproline output was measured with the subjects on a gelatin-free diet for 48 hours before and during the collections by a modification (7) of the method of Prockop and Udenfriend (8). The mean daily output over a period of at least 4 days was determined. Blood for serum alkaline phosphatase activity was measured by the method of Bessey, Lowry, and Brock (9) at 3-day intervals during a hospital investigation of approximately 2 weeks, and the highest recorded values were utilized in the analysis of the results.

Eighty- to eighty-five-day-old rats of the Sprague-Dawley strain were divided into five groups. Group 1 served as untreated controls. Groups 2, 3, 4, and 5 received 1, 2, 3, and 4 daily doses of 200 U of parathyroid extract (PTE) [1] by subcutaneous injection, the last dose in each case being given 16 to 18 hours before death. The administration of excess hormone in the presence of intact parathyroids was considered to provide the model that most closely approximates the human disease.

After death by decapitation animal bone samples were taken from the upper tibial and lower femoral metaphyses as previously described (10). These and the human samples removed at biopsy were similarly collected into chilled (2 to 4° C) Krebs-Ringer medium, buffered with bicarbonate at pH 7.4, and maintained at this temperature

[1] Eli Lilly Co., Indianapolis, Ind.

TABLE I

*Pertinent clinical data for the patients reported**

Patient	Age	Sex	Complaints	Serum Ca	Serum P	Serum Alkaline phosphatase	24-hour urine output Ca	24-hour urine output Hydroxyproline	X rays	Pathologic diagnosis
	years			*mEq/L*	*mμ moles/L*	*B-L units*	*mg*	*mg*		
				"Continuous" group						
R. C.	50	F	Peptic ulcer	5.9	0.9	4.2	420	259	Typical changes in skull and phalanges	Parathyroid carcinoma
E. D.	41	F	Renal stone Weight loss Anorexia Mass in neck	10.1	1.4	4.0	700	197	Typical changes in skull and hands	Parathyroid adenoma (15 g in wt)
D. S.	51	M	Peptic ulcer Polyuria Polydipsia Weight loss Uremia Nephrocalcinosis upon biopsy	6.6	0.5	4.7	291	57	Osteoporosis Rarefaction Left lower femoral metaphysis	Generalized parathyroid hyperplasia
R. W.	36	M	Uremia Pain in knee	6.5		4.7			Cyst of right patella with fracture	Osteitis fibrosa cystica (no neck exploration)
W. M.	32	M	Terminal uremia with kidney transplant Limb pain Metastatic calcification in shoulder and hand	7.0	4.6	4.5			Rarefaction in hands and feet Metastatic calcification	Generalized parathyroid chief cell hyperplasia
				"Intermittent" group						
C. M.	50	F	Duodenal ulcer Renal stone	6.2	1.0	2.2	304	13.9	Negative	Right lower parathyroid adenoma
M. C.	45	F	Duodenal ulcer Renal stone	5.6	1.0	1.4	141	21.8	Negative	Right lower parathyroid adenoma
M. N.	78	F	Constipation Weight loss Confusion	6.6	1.0	2.7		29.4	Negative	Right lower parathyroid adenoma

* The patients have been divided into a "continuous" and an "intermittent" group (see Results section of text for details). Note that seven of the eight had surgically proven parathyroid disease; one had changes in bone histology characteristic of hyperparathyroidism. Normal ranges for the values cited are as follows: serum calcium, 4.5 to 5.5 mEq per L; serum phosphorus, 0.9 to 1.2 mmoles per L; serum alkaline phosphatase, 0.6 to 2.3 Bessey-Lowry units per 100 ml (males), 0.5 to 1.9 Bessey-Lowry units per 100 ml (females). Twenty-four-hour urinary hydroxyproline output in adults on a gelatin-free diet was 24.7 ± 2.5 mg. The upper limits of normal 24-hour urinary calcium output after 1 week on a 200-mg calcium intake is 150 mg per day.

throughout the preparative procedures. The subsequent handling of the tissue in preparation for incubation, the conditions of incubation, the collection of the various fractions, the analyses, and the calculation of results were carried out as previously described (1, 2, 6, 10).

Results

The initial assumption was made in these experiments that bone metabolism in hyperparathyroid patients would differ from that of normal adults, but no consistent pattern of change was found upon first examination. Closer scrutiny, however, revealed that patients could be divided into two groups, one characterized by greatly increased bone metabolic activity, and the other by normal or decreased activity. For reasons that will be brought out in the Discussion, we have chosen to regard the former as indicative of continuous hypersecretion, and the latter as indicative of intermittent hypersecretion, of parathyroid hormone. The data are presented in this fashion in Figures 1 through 6, together with the previously established values for normal adult human bone for comparison (1).

The changes in bone cell oxygen uptake in the two groups of subjects in this study are presented in Figure 1. The intermittent group showed a mean increase of approximately 80% above normal. This difference did not quite reach acceptable levels of statistical significance. The continuous group showed an increase of approximately 160% above the normal level, a difference that was significant at $p < 0.01$.

The mean rates of lactate production by bone from the two groups are shown in Figure 2. Lactate production in the intermittent group was

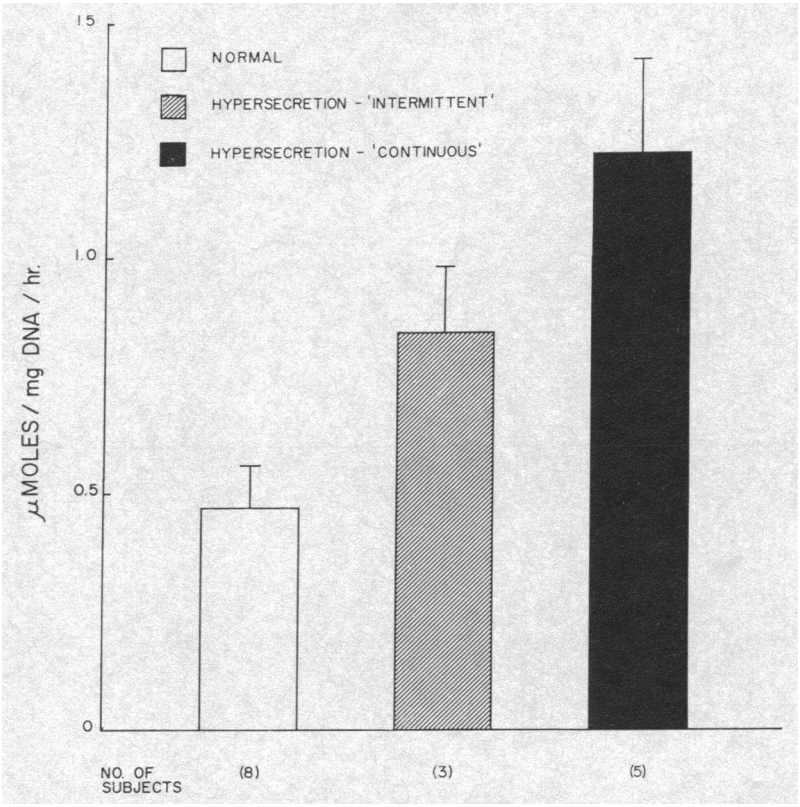

FIG. 1. MEAN OXYGEN UPTAKE OF HUMAN BONE FROM NORMAL PATIENTS AND THE TWO GROUPS OF PATIENTS WITH HYPERPARATHYROIDISM. The magnitude of 1 SE of each mean is indicated in this and subsequent Figures by the vertical line extending above each bar. Individual values were calculated from the total O_2 uptake of the bone during 2 to 4 hours. See Discussion section of text for details on "intermittent" and "continuous" groups.

normal, whereas the continuous group showed a highly significant threefold elevation of lactate production above the normal range ($p < 0.01$).

Rates of proline incorporation into the cell fraction and collagen of the bone samples are shown in Figure 3. As has been observed previously the labeling in the cells was about 100 times greater than in the collagen (1). The intermittent group again showed no significant difference from normal with respect to incorporation into the cells, whereas the continuous group showed a marked mean elevation of incorporation. The wide variation in the values in this group (note the large standard error) prevented the difference from reaching an acceptable level of statistical significance; however, it should be noted that all the individual values were at least double the mean normal value.

The rate of incorporation of proline into collagen, on the other hand, showed very striking and opposing changes. The intermittent group showed a mean incorporation rate decreased to approximately 40% of the normal value, which was significant at $p < 0.02$, whereas the continuous group showed a sevenfold mean increase in incorporation rate over the normal level, which was significant at $p < 0.05$. Again it was noteworthy that despite wide variations in the values in hyperparathyroid groups, all were clearly different from the mean normal value.

Glucose incorporation rates for bone from the three groups are presented in Figure 4. The changes in incorporation into both cells and collagen generally resembled those found for proline in the corresponding intermittent and continuous groups, although the clear depression of collagen

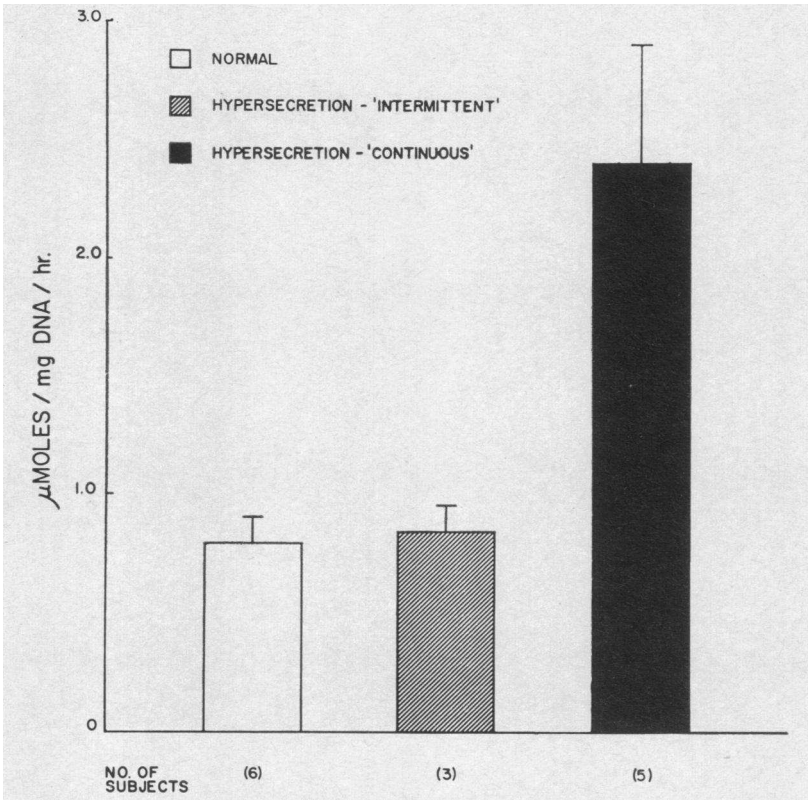

FIG. 2. RATES OF LACTATE PRODUCTION BY BONE FROM THE TWO HYPERPARATHYROID GROUPS COMPARED TO NORMAL SUBJECTS. Individual values were derived from the release of lactate (measured chemically) into the incubation medium during 2 to 4 hours of incubation. Each represents the mean of at least two incubations of bone from the same subject.

labeling from proline in this intermittent group was absent when glucose was the labeled substrate. However, none of the changes even in the continuous group reached statistically acceptable levels because of the very wide scatter of the values, a phenomenon perhaps related to varying endogenous glucose stores from sample to sample.

The total 24-hour excretion of hydroxyproline in the urine was measured in these patients and compared to that of normal ones. The mean values observed in the two hyperparathyroid groups are compared with the mean values of a group of seven normal adult subjects in Figure 5. The intermittent hypersecretion group showed no significant difference from the normal group, whereas the continuous hypersecretion group showed a fivefold mean increase over the normal value, the difference being significant at $p < 0.01$.

These changes correlated closely with the alterations in serum alkaline phosphatase levels found in these patients, which are presented in Figure 6. Again the intermittent group showed no deviation from normal, whereas the continuous group showed a mean threefold increase above the normal range.

An inhibition of proline incorporation into collagen such as was seen in the intermittent group has been shown to be an early effect of a single dose of parathyroid hormone (6) rather than a stimulation such as appeared in the continuous group. It became of interest, therefore, to determine whether the increases in collagen biosynthesis in bone that had been shown to follow as a secondary phenomenon after a single dose of PTE (4, 5) also developed during continuous hormone administration. The effects of multiple

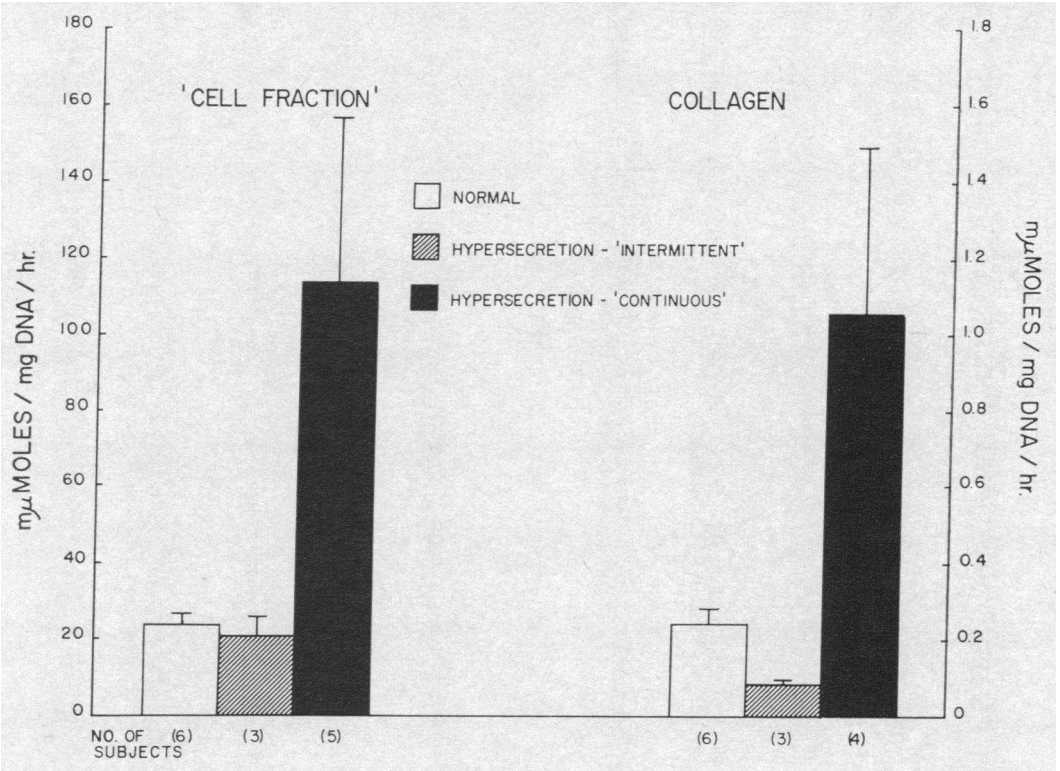

FIG. 3. RATES OF PROLINE INCORPORATION INTO THE CELL FRACTION AND COLLAGEN OF BONE SAMPLES FROM THE THREE GROUPS. Data were calculated from the over-all uptake of ^{14}C label from the medium over 3 to 4 hours of incubation and have been expressed in terms of millimicromoles of proline in order to eliminate differences due to differences in medium proline specific activity. Note that the ordinate scale on the left is for the cell fraction, whereas the one on the right with units 100 times smaller refers to the collagen.

daily doses of PTE on these phenomena were therefore investigated in rats.

Data from a typical set of experiments are shown in Figure 7, which contrasts the effects of PTE treatment on proline incorporation into collagen and lactate production. It can be seen that the depression of collagen synthesis noted in previous experiments was apparent again after 1 and 2 days of treatment. However, by the third and fourth days this inhibition had either disappeared or had been overwhelmed by a secondary stimulation of the synthetic rate. It was particularly interesting to note that the single dose that caused a depression of synthesis on the first day was quite ineffective in suppressing the increased synthesis, which had begun to appear by 3 and 4 days. Although the timing of these effects varied somewhat from experiment to experiment, both the initial inhibition of collagen synthesis and the later stimulation proved statistically significantly different from controls, $p < 0.02$ and < 0.05, respectively, when data from several experiments were pooled. In contrast to these changes in proline incorporation into collagen no depression of lactate production occurred. Instead a continuously increasing rate of production was noted with continuing treatment. This increase had become statistically significant by the third day of treatment.

In contrast to the apparent response of human bone that these experiments were designed to duplicate, no consistent changes in bone oxygen consumption and glucose uptake were demonstrable under the dosage schedules used.

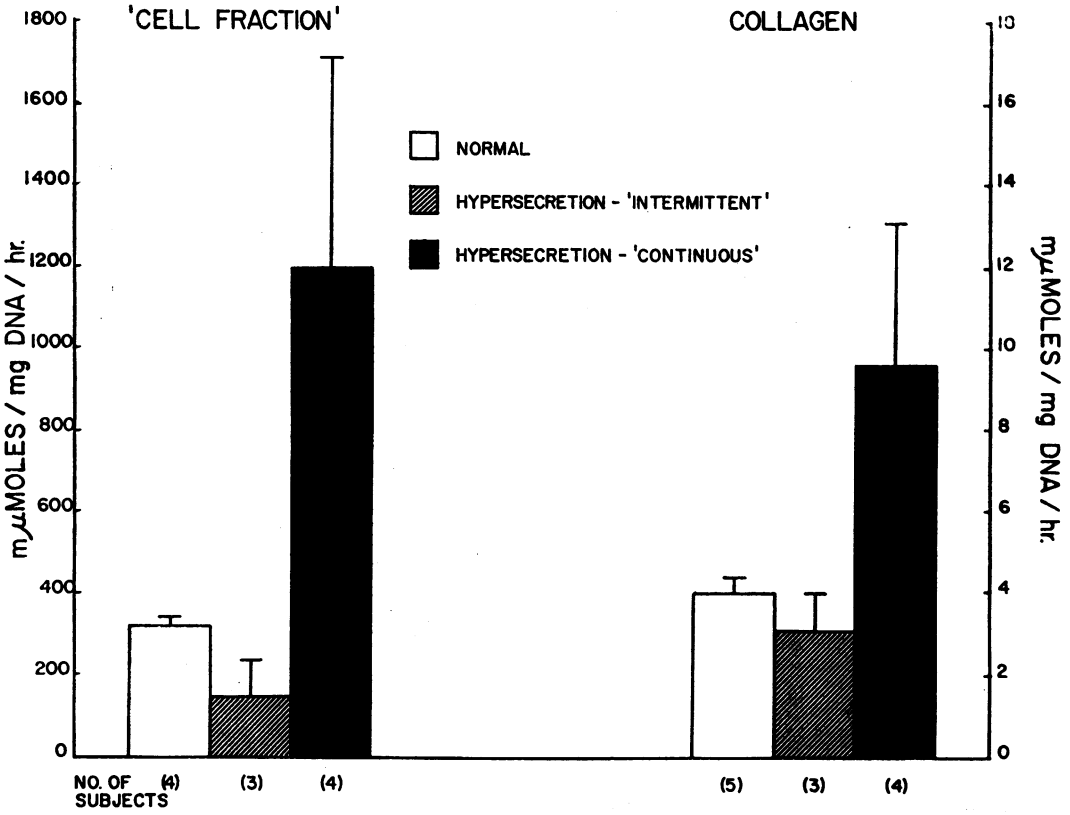

FIG. 4. INCORPORATION OF GLUCOSE INTO CELLS AND COLLAGEN IN BONE FROM NORMAL SUBJECTS AND THE TWO HYPERPARATHYROID GROUPS. Data have been calculated and expressed as in Figure 3. Note that the scale for cell label is on the left and the one for collagen is on the right. Again the units in the former are 100 times the latter. Both are 10 times those for proline, reflecting the proportionately greater incorporation of glucose into the tissue.

Discussion

The results presented here indicate that two distinct types of abnormal bone metabolism appear to exist in hyperparathyroidism. Moreover, when patients are separated into two groups on the basis of such *in vitro* metabolic data alone, the members of each group turn out to have common clinical characteristics as well.

Bone metabolism in the continuous group was characterized by increased activity, often very striking, in all the metabolic systems measured. These patients exhibited the majority of the usual clinical signs and symptoms of hyperparathyroidism. Hypercalcemia and elevated serum alkaline phosphatase levels, hypercalciuria and increased total 24-hour urinary hydroxyproline excretion, and some X-ray evidence of bone disease were present in all. Symptoms were more variable, but all gave one or more complaints suggesting hypercalcemia either present at the time of study or troublesome in the recent past.

In contrast, bone from the intermittent group was metabolically normal with two exceptions: oxygen consumption tended to be elevated, and the incorporation of proline into collagen was significantly decreased. This pattern was found in bone from those patients who were distinguished by the paucity of their manifestations of parathyroid disturbance. X-ray evidence of bone disease was notably absent, and hypercalciuria was either minimal or only intermittently present. Moreover, serum Ca and P concentrations were borderline or normal, whereas serum alkaline phosphatase activity was not elevated, and urine hydroxyproline excretion was in the normal range. Indeed, the diagnosis had to be made in

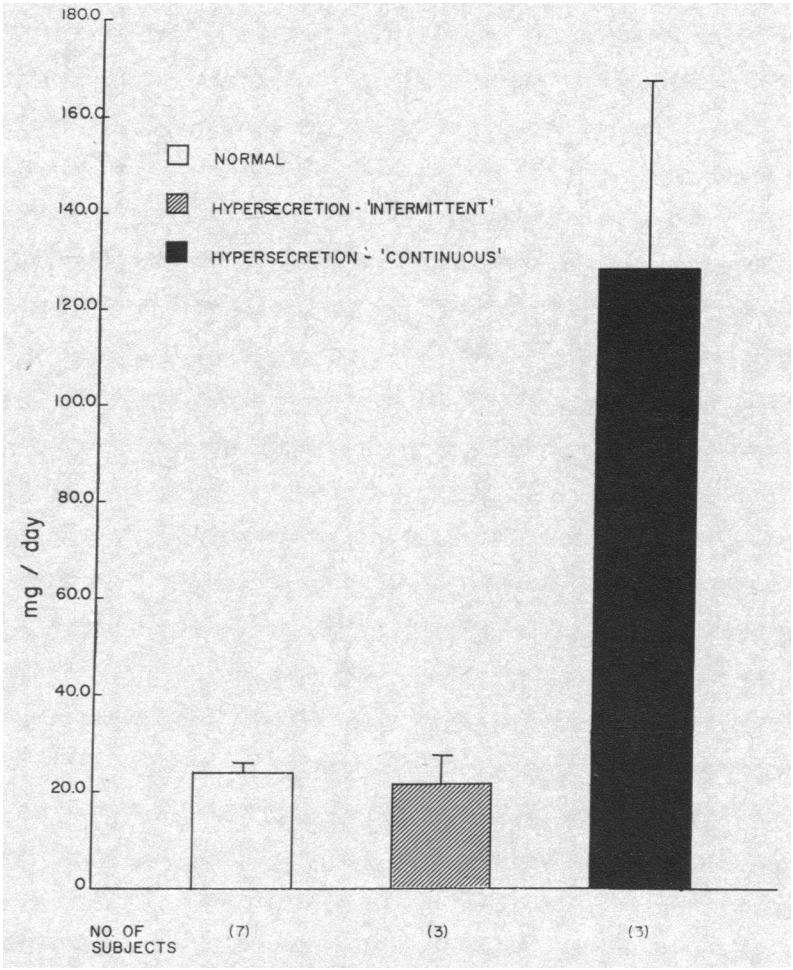

FIG. 5. MEAN TOTAL 24-HOUR URINE HYDROXYPROLINE CONTENT IN THE TWO GROUPS OF HYPERPARATHYROID SUBJECTS AND SEVEN ADULTS WITHOUT BONE OR KIDNEY DISEASE.

these patients on the basis of recurrent kidney stone formation, minimal, often intermittent, hypercalcemia or hypercalciuria, and abnormal response to tests of the control of parathyroid function, or all of these symptoms (11, 12).

The demonstration of these two distinct patterns of response raises the question of whether the difference between these two groups is a quantitative or a qualitative one, a question made more pertinent by Costello and Dent's recent suggestion that there may exist a separate hormone responsible for the changes of osteitis fibrosa cystica (13). Although the data presented here cannot exclude the existence of two different hormones acting on bone or, indeed, of two differing tissue responses to the same hormone, they do not seem to necessitate such postulates.

All of the bone metabolic changes observed in these samples, with the possible exception of the increased oxygen consumption, have now been observed in animals treated with PTE under various schedules (4–6). The only question that remained open is, can the increase in collagen biosynthesis that follows secondarily after the initial inhibition caused by PTE occur even if repeated doses of hormone are given. It appears to be answered affirmatively by the animal experiments reported above.

On the basis of these findings it seems reasonable for the present to regard the two different

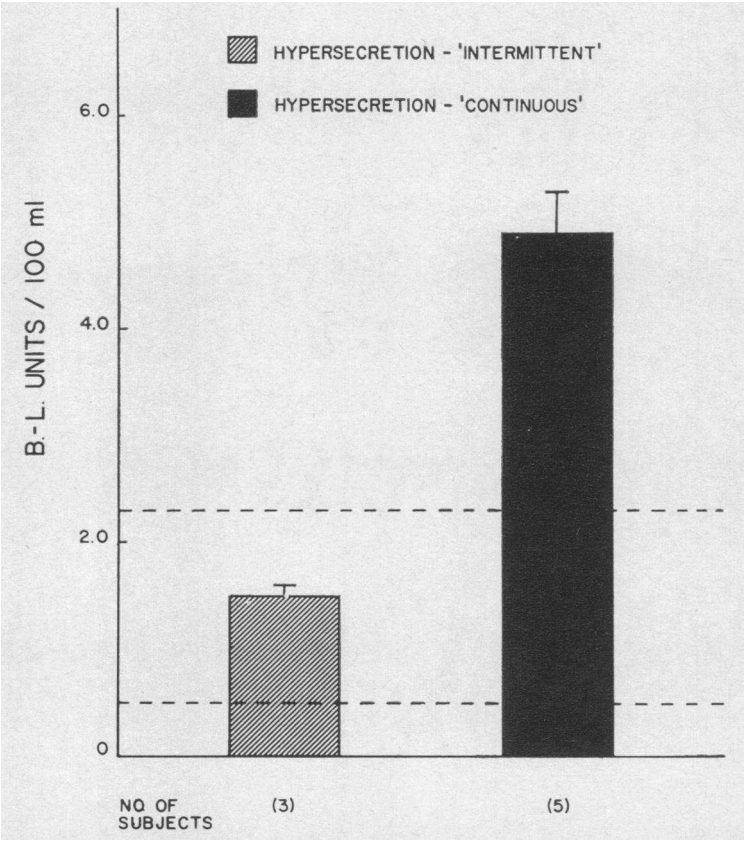

FIG. 6. MEAN PLASMA ALKALINE PHOSPHATASE ACTIVITY MEASURED IN BESSEY-LOWRY UNITS IN THE TWO GROUPS OF HYPERPARATHYROID PATIENTS. The limits of the normal range in this laboratory are shown by the two dotted lines.

responses seen in the human subjects as variations in the intensity or degree of disturbance of parathyroid function. As the disease involves not only hypersecretion of the hormone but also loss of secretory control in the abnormal gland or glands (11, 14), this hypothesis may be taken one step further to suggest an explanation for the appearance of two types of bone metabolic response in these patients, an explanation which, incidentally, provided the names "intermittent" and "continuous" used in this work to designate the two groups.

One group, according to this view, will contain those patients in whom the sum of the secretion from the normal and abnormal parathyroids only intermittently exceeds normal. This can theoretically occur since the secretory rate of the abnormal gland, while excessive and uncontrolled, also fluctuates, as is attested to by the fluctuating hypercalcemia clinically seen in patients with parathyroid adenomata. Thus total secretion would be excessive at times when the output of the abnormal gland was greater than could be compensated for by reduction of the secretion of the normal glands.[2] The animal work implies that bone from such individuals should exhibit only the acute effects of hormone-increased lactate production (2, 3) and an inhibition of proline incorporation (5, 6), whereas clinical considerations suggest that these patients should exhibit minimal symptoms and signs of the disease. The other group will then include all patients with more active abnormal glands in whom the level of hor-

[2] According to this hypothesis, the intermittent group should theoretically include only patients with adenoma, as is borne out by the surgical findings (Table I).

monal secretion is continuously above normal. In such individuals bone metabolic patterns should resemble those seen in the animals who had received the maximal number of doses of PTE. Similarly, the clinical manifestations of the disease would be expected to be most florid in this group. The high degree to which the clinical manifestations of the disease in the two groups coincided with these predictions is indicated by the data in Table I.

The observations regarding lactate production are in keeping with the findings in all animal studies including the present one. The absence of a significant elevation in the intermittent group would imply that the stimulus was either not sufficiently prolonged or severe to produce it. Similarly, the failure to reproduce the increased oxygen utilization in the animal model while at the same time producing the other changes found in the human situation suggests that a more chronic degree of stimulation of the bone cells may also be required to produce this phenomenon.

The association of the elevation of serum alkaline phosphatase and urinary hydroxyproline

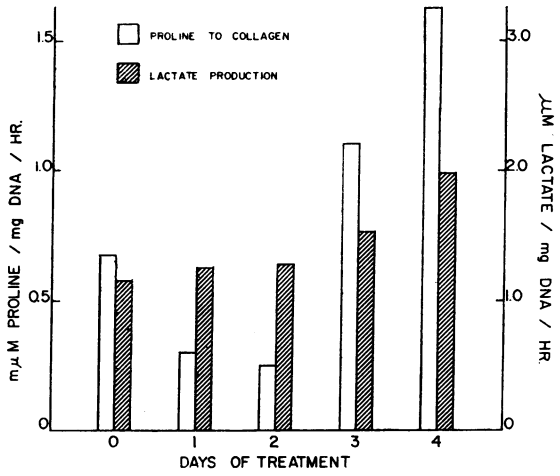

FIG. 7. CHANGES IN RATES OF PROLINE INCORPORATION INTO COLLAGEN AND LACTATE PRODUCTION IN BONE FROM ANIMALS TREATED FOR INCREASING PERIODS WITH DAILY INJECTIONS OF PARATHYROID EXTRACT IN A TYPICAL EXPERIMENT. Each bar in the case of proline represents one, or the mean of two or three, separate incubations of samples taken from a single pool of bone derived from two to four rats. In the case of lactate each bar represents the means of three to four samples from the same pool. Note that the scale on the left ordinate is for proline, whereas the one on the right is for lactate.

levels in this disease has previously been described. Both are regarded as sensitive clinical indexes of bone involvement; increased alkaline phosphatase is associated with an increase in osteoblastic activity (14), and increased urinary hydroxyproline is associated with an increase in either collagen synthesis or breakdown or both (7, 11).[3] The results of the *in vitro* metabolic bone studies reported here suggest that some abnormality of bone metabolism exists in this disease even in the absence of clinical evidence of bone involvement by these indexes. The fact that the abnormality observed under these conditions did not involve any stimulation of new collagen biosynthesis may be in some way related to this absence of change.

The ultimate value of metabolic studies of bone *in vitro* as a tool in the diagnosis of hyperparathyroidism remains to be proven. At present, the procedure remains too traumatic and the techniques too complex for routine clinical use. However, in two patients (M.C., C.M.) results obtained at biopsy did prove useful in establishing the diagnosis preoperatively. It must be pointed out that the biochemical lesions in bone cannot yet be called pathognomonic of an excess of parathyroid hormone. If and when such lesions can be established as pathognomonic and if, as seems likely from unpublished experiments, the methods can be adapted to studying much smaller fragments of bone, then these measurements may prove to be the most rapid and certain means of diagnosing hyperparathyroidism yet devised.

Summary

1) Two types of metabolic change found *in vitro* in bone from hyperparathyroid patients have been presented.

2) These are interpreted as manifestations of differing degrees of overactivity of the parathyroids, one group reflecting intermittent, and the other continuous, hypersecretion of parathyroid hormone.

3) The intermittent group is characterized by a depression of proline utilization for collagen

[3] The findings in patient E.D., the most florid case of all, are of interest with regard to the source of the urinary hydroxyproline in this disease. After removal of the adenoma, the 24-hour urinary hydroxyproline output fell from 197 mg in one day to the normal range.

synthesis occurring with a modest increase in oxygen consumption.

4) The continuous group is characterized by larger increases in oxygen utilization, increased lactate production, and increased utilization of proline for collagen synthesis together with raised levels of serum alkaline phosphatase and urinary hydroxyproline.

5) The significance of these changes is discussed in relation to the actions of the hormone and to the clinical presentation and diagnosis of the disease.

6) Some partial support for this hypothesis is provided by animal experiments.

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