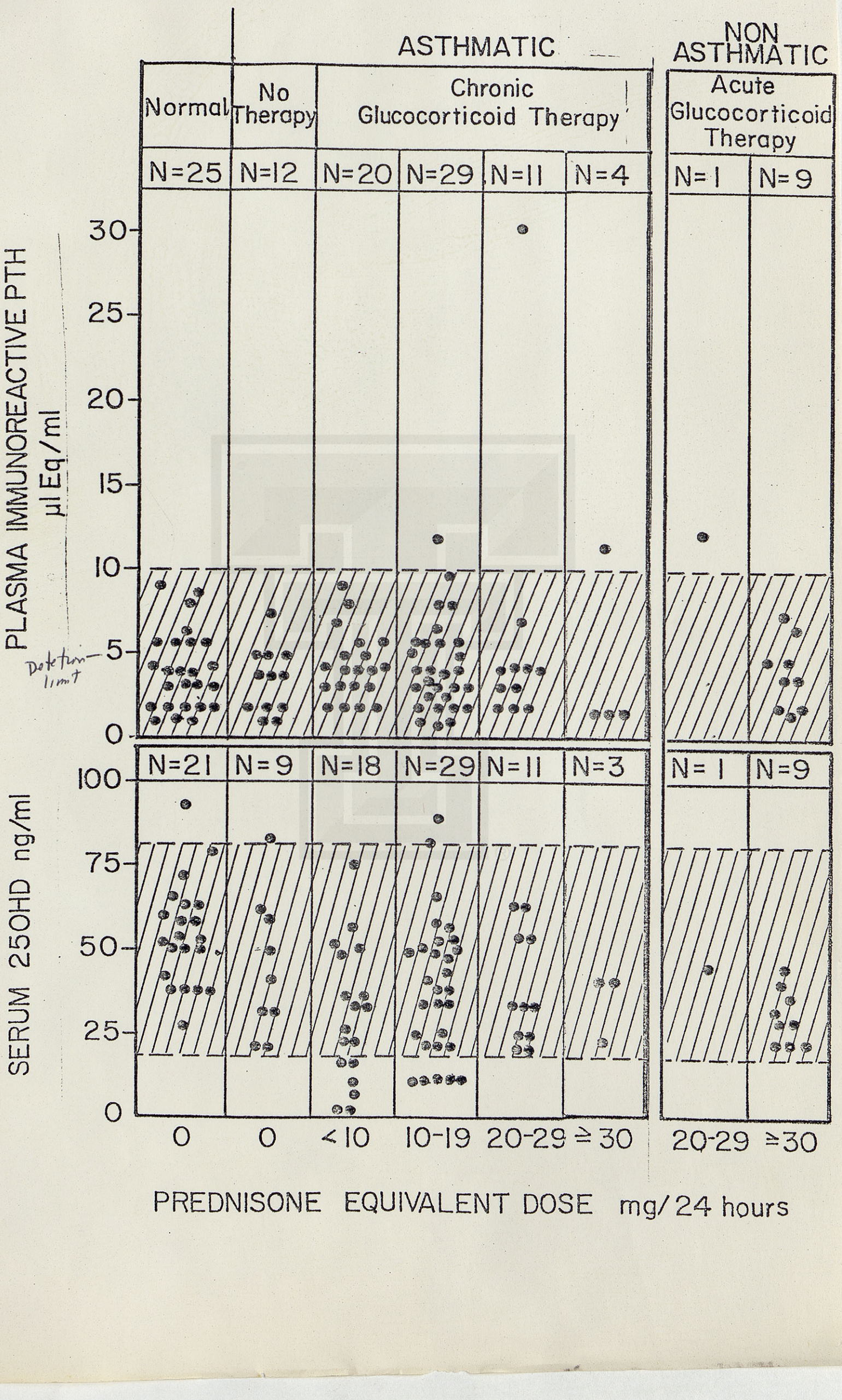
ASTHMATIC
NON ASTHMATIC
Normal
No Therapy
Chronic Glucocorticoid Therapy
Acute Glucocorticoid Therapy
N=25
N=12
N=20
N=29
N=11
N=4
N=1
N=9
PLASMA IMMUNOREACTIVE PTH μl Eq/ml
30
25
20
15
10
5
0
Detection limit
N=21
N=9
N=18
N=29
N=11
N=3
N=1
N=9
SERUM 25OHD ng/ml
100
75
50
25
0
0
0
<10
10-19
20-29
≥30
20-29
≥30
PREDNISONE EQUIVALENT DOSE mg/24 hours

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

RESEARCH AND TECHNOLOGY RESUME

1. DATE PREPARED: 4/25/77

2. TITLE: Investigations in Hormonal Control of Calcium and Bone Metabolism

3. NUMBER/CODE: a. PROPOSAL: | b. CURRENT: 199-07-01-13

4. PERFORMING ORGANIZATION:
Massachusetts General Hospital
Fruit Street
Boston, Massachusetts 02114

5. CONTRACT/GRANT NO.: NAS 9-15204

6. DATE: a. STARTING: 1-77 | b. ANNIVERSARY: 12-77

7. INVESTIGATOR'S NAME: Robert M. Neer, M.D. | TEL. NO.: 617/726-3966

8. NASA ALT. TECH MONITOR'S NAME | TEL. NO.:

9. INSTITUTION CATEGORY CODE: 0

FISCAL YEAR	STATUS a.	MANPOWER (MY) IN-HOUSE b.	MANPOWER (MY) S/C c.	FUNDING (In K) IMS d.	FUNDING (In K) R/D e.	FUNDING (In K) TOTAL f.
10. PRIOR	C	.1	.0	0	20	20
11. CURRENT	C	.1	1	32	70	102
12. BUDGET	C	.1	1	32	75	107

13. DESCRIPTION *(a. Brief statement on strategy of investigation; b. Progress and accomplishments of prior year; c. What will be accomplished this year, as well as how and why; and d. Summary bibliography)*

a. Strategy:
This combined program of investigations in normal volunteers and continuing studies in the development of biochemical techniques is designed to add depth in the bone and muscle area by consideration of the biochemical changes which occur in conditions of calcium loss.

b. Progress and Accomplishments of Prior Year:
Significant improvements were obtained in the sensitivity, specificity, and methodology in the assays for calcitonin. Several antibodies have been developed through immunizations with human calcitonin in chickens; the antibodies have extremely high sensitivity. These antisera appear to provide the most sensitive immunoassays for calcitonin yet developed by any laboratory. One antibody (APC-15-25) can be used in final dilutions in the assay of greater than 1-100,000; the sensitivity for detection appears to be considerably less than 50 pg/ml. This level of sensitivity permits easy measurement of the calcitonin level circulating in all normal human subjects. In the last several years there has been increasing attention directed to the probability that calcitonin is circulating, but at very low levels, in normal subjects. The analysis of the dynamics of control of calcitonin production in normal individuals, however, has required antisera of much greater sensitivity than those used recently for detection of calcitonin production by medullary carcinoma; in the latter subjects, concentrations of 500-750 pg/ml up to the range of several thousand pg/ml or higher circulate.

A second development is an alternate approach of hormone assay based upon the adoption of the recently described bacteriophage assay technique as a substitute for conventional radioimmunoassay. This assay is based upon inhibition of the infection and lysis of bacteria by bacteriophage due to the presence of hormone convalently labeled to the tail fibers of the phage. The principle of the assay is that the presence of the labeled antigen on the tail fibers makes them susceptible to loss of infectivity when they are incubated with antibody which cross links the tail fibers. Prior exposure of the antibody to standard or test solutions containing calcitonin neutralizes antibody's ability to bind to the antigen on the tail fiber of the

	TYPED NAME AND SIGNATURE	DATE
TECHNICAL MONITOR	Carolyn S. Leach, Ph.D.	4-26-77
APPROVING OFFICIAL	Richard S. Johnston	

NASA FORM T43 DEC 74 REPLACES NASA FORM T8 WHICH IS OBSOLETE.

phage and, thereby, no longer inhibits the infection of the bacteria by the bacteriophage and the subsequent lysis of the bacteria. This technique provides even greater sensitivity for a given antibody than can be accomplished with traditional solution phase radioimmunoassays. Although the precise details of the assay have not been worked out it seems likely that levels in the range of 1 pg/ml might be measured.

Many new antisera are also being developed for parathyroid hormone. Success in the determination of the sequence of the hormone followed by synthesis of relevant regions of the human parathyroid hormone molecule continues. It seems likely that the advances made in the bacteriophage immunoassay approach will be applicable to improved sensitivity and simplicity in work with parathyroid hormone immunoassay.

Nonequilibrium conditions have been adapted to conventional assay for 25-hydroxy D and it was found that the sensitivity can be greatly increased by a factor of 3-to-4-fold. Synthetic efforts in this laboratory have been successful in the preparations of radioactive 1,25-dihydroxy D to be used as tracer in a protein binding assay for the estimation for this critical metabolite in the blood. Although the assay for 1,25-dihydroxy D is not yet completely reduced to practice and operation on plasma samples from normal subjects and those with various disease states, the rapid recent advances in the techniques for separation of the various metabolites from each other, the introduction of the high specific activity 1,25-dihydroxy D tracer and other improvements in the general methodology show that a highly useful and functioning assay for the biologically interesting metabolites of vitamin D will be accomplished.

c. Objectives;

1. Biochemical techniques to be addressed include;

   a. Improved Assays for Parathyroid Hormone

      (1) Continue efforts to analyze chemical features and biological control of glandular products such as pre-proparathyroid hormone, the prohormone hexapeptide, proparathyroid hormone and parathyroid secretory protein so that by isolation or synthesis specific immunoassays can be developed for their measurement.

      (2) Develop a radioreceptor assay for PTH.

      (3) Develop and apply the radioimmunoassays for the glandular products and, as well, for synthetic fragments of PTH such as the biologically-active amino-terminal region to determine their discriminant value in normal physiology and disorders of calcium metabolism.

   b. Improved Assays for Vitamin D and Active Metabolites

      (1) Develop a sensitive and specific immunoassay for 1,25-$(OH)_2D$ by use of synthetic 1,25-$(OH)_2D$ and the plasma binding protein for vitamin D metabolites.

(2) Determine production rates of 1,25-$(OH)_2D$ in normal and abnormal states of calcium metabolism.

c. Improved Assays for Calcitonin

(1) Develop immunoassays for synthetic fragments of calcitonin to provide more sensitive and region-specific assays.

(2) Apply the immunoassays and detailed analyses of gland extracts, gland effluent and peripheral blood to determine the best method for estimating secretion of biologically-active hormone.

2. The new part of this effort will cover studies of bone resorption and calcium metabolism in normal volunteers and patients. Stable calcium isotopes will be used to measure skeletal calcium resorption in man, and comparative study will define the most sensitive method for measuring small changes in skeletal resorption. This study will be carried out in normal volunteers, with measurements made under controlled conditions before and during the administration of synthetic human parathyroid hormone in doses just sufficient to increase bone resorption by a physiological amount. The stable isotope dilution method for measuring skeletal calcium resorption in man is applicable to sensitive studies of bone turnover during space flight, but needs additional validation and comparative testing.

Further efforts will be directed toward the study of intestinal calcium absorption, and the hormonal signals regulating calcium metabolism, in humans receiving glucocorticoids. Since a 1.5-2 fold excess of endogenous glucorcorticoid production is characteristic of prolonged space flight, studies in normal volunteers acutely given comparable doses of exogenous glucorcorticoid, and patients chronically receiving similar doses of exogenous glucocorticoid excess in man of recently reported abnormalities in parathyroid hormone levels and vitamin D metabolism in animals receiving large doses of glucocorticoids. Therapeutic studies to prevent calcium loss be administering synthetic 1,25$(OH)_2D_3$ to humans with chronic glucocorticoid excess will be undertaken as well, if the initial pathyophysiological studies indicate that this can reverse the glucocorticoid induced abnormalities in calcium metabolism in man to the same degree as claimed for animal studies. Chronic glucocorticoid excess is believed to be responsible for some of the abnormalities in calcium metabolism during space flight.

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CABLE ADDRESS "MASSGENRAL"

TO: National Aeronautics and Space Administration

Principal Investigator: Robert M. Neer, M.D.

Title of Project: Hormonal Interactions in Bone Resorptive Processes

Total Amount Requested: $146,367

Dates: 1977—1980

10/12/76
Date

Charles A. Sanders, M.D.
General Director

SBR-13-01-07

HORMONAL INTERACTIONS IN BONE RESORPTIVE PROCESSES

Robert M. Neer, M.D.
Principal Investigator

C U R R I C U L U M V I T A E

Robert Marshall Neer

Born:	November 12, 1935, Celina, Ohio
1957	B.A. Cum laude Harvard College, Cambridge, Massachusetts
1961	M.D. Columbia College of Physicians and Surgeons, New York City
1961-62	Intern in Medicine, Bronx Municipal Hospital Center, New York
1962-63	First Year Medical Resident, Bronx Municipal Hospital Center
1963-65	Clinical Associate, Metabolism Branch, National Cancer Institute, Bethesda, Maryland
1965-66	Resident in Medicine, Department of Medicine, Yale-New Haven Hospital, New Haven, Connecticut
1966-68	Clinical and Research Fellow in Endocrinology, Massachusetts General Hospital, Boston, Massachusetts
1968-	Associate Director, Metabolic Research Center, Massachusetts General Hospital
1968-71	Assistant in Medicine, Massachusetts General Hospital
1968-71	Instructor in Medicine, Harvard Medical School
1971-	Assistant Professor of Medicine, Harvard Medical School
1971-74	Assistant Physician, Massachusetts General Hospital
1974-	Associate Physician, Massachusetts General Hospital

Memberships:

1960	Alpha Omega Alpha
1966	American Federation for Clinical Research
1967	Diplomate, American Board of Internal Medicine

P U B L I C A T I O N S

1. Neer, R.M., Zipkin, I., Carbone, P. and Rosenberg, L.E.: Effect of sodium fluoride therapy on calcium metabolism in multiple myeloma. J. Clin. Endocrinol. Metab. 26:1059-1068, 1966.

2. Neer, R., Berman, M., Fisher, L. and Rosenberg, L.E.: Multi-compartmental analysis of calcium kinetics in normal adult males. J. Clin. Invest. 46: 1364-1379, 1967.

3. Phang, J., Berman, M., Finerman, G.A., Neer, R.M., Rosenberg, L.E. and Hahn, T.J.: Dietary perturiation of calcium metabolism in normal man: Compartmental analysis. J. Clin. Invest. 48:67-86, 1969.

4. Judd, H.L., Scully, R.E., Atkins, L., Neer, R.M. and Kliman, B.: Pure gonadal dysegenesis with progressive hirsutism. New Eng. J. Med. 282:881-885, 1970.

5. Neer, R.M., Potts, J.T., Parsons, J.A., Krane, S.M., Deftos, L.J. and Shields, C.L.: Pharmacology of calcitonin: Human Studies. In, Calcitonin 1969, Proceedings of the 2nd International Symposium. ed. S. Taylor and G. Foster, Heinemann, London, pp. 547-554, 1970.

6. Singer, F.R., Neer, R.M., Murray, R.M., Keutmann, H.T., Deftos, L.J. and Potts, J.T., Jrs.: Meithramycin treatment of intractable hypercalcemia due to parathyroid carcinoma. New Eng. J. Med. 283:634-644, 1970.

7. Habener, J.F., Singer, F., Deftos, L., Neer, R.M. and Potts, J.T., Jr.: Explanation for the unusual potency of salmon calcitonin. Nature [New Biol] 232:91-92, 1971.

8. Neer, R.M., Davis, T.R.A., Walcott, A., Koski, S., Schepis, P., Taylor, I., Thorington, L. and Wurtman, R.J.: Stimulation by artificial lighting of calcium absorption in elderly human subjects. Nature 229:255-257, 1971.

9. Parsons, J.A., Neer, R.M. and Potts, J.T.: Initial fall of plasma calcium after intravenous injection of parathyroid hormone. Endocrinology 89: 735-740, 1971.

10. Habener, J.F., Singer, F.R., Neer, R.M., Deftos, L.J. and Potts, J.T., Jr.: Metabolism of salmon and porcine calcitonin: An explanation for the increased potency of salmon calcitonin. In, Calcium, Parathyroid Hormone and the Calcitonins. ed. R.V. Talmage and P.L. Munson, Excerpta Medica, Amsterdam, pp. 152-156, 1972.

11. Singer, F.R., Aldred, J.P., Neer, R.M., Krane, S.M., Potts, J.T., Jr. and Bloch, K.J.: An evaluation of antibodies and clinical resistance to salmon calcitonin. J. Clin. Invest. 51:2331-2338, 1972.

12. Singer, F.R., Keutmann, H.T., Neer, R.M., Potts, J.T., Jr. and Krane, S.M.: Pharmacological effects of salmon calcitonin in man. In, Calcium, Parathyroid Hormone and the Calcitonins. ed. R.V. Talmage and P.L. Munson, Excerpta Medica, Amsterdam, pp. 89-96, 1972.

13. Deftos, L.J. and Neer, R.M.: Medical management of the hypercalcemia of malignancy. Annual Review of Medicine 25:323-331, 1974.

14. Singer, R.F., Neer, R.M., Goltzman, D., Krane, S.M. and Potts, J.T., Jr.: Treatment of Paget's disease of bone and hypercalcemia with salmon calcitonin. In, Endocrinology 1973: Proceedings of the Fourth International Symposium. London, July 8-13, 1973. ed. S. Taylor, William Heinemann Medical Books Limited, London, pp. 397-408, 1974.

15. Neer, R.M.: The evolutionary significance of vitamin D, skin pigment, and ultraviolet light. Am. J. Physical Anthropol. 43:409-416, 1975.

16. Neer, R.M., Holick M.F., DeLuca, H.F. and Potts, J.T., Jr.: Effects of 1α-OH vitamin D_3 and 1,25-$(OH)_2$ vitamin D_3 on calcium and phosphorus metabolism in hypoparathyroidism. Metabolism 24:1403-1413, 1975.

17. Bigos, S.T., Neer, R.M., St. Goar, W.T.: Hypercalcemia of seven years' duration after kidney transplantation. Am. J. Surgery 132:83-89, 1976.

18. Delmonico, F.L., Neer, R.M., Cosimi, A.B., Barnes, A.B., Russell, P.S.: Hyperparathyroidism during pregnancy. Am. J. Surgery 131:328-337, 1976.

ABSTRACTS

1A. Neer, R.M., Davis, T.R. and Thorington, L.: Use of environmental lighting to stimulate calcium absorption in healthy men. Clin. Res. 18:693, 1970.

2A. Neer, R.M., Belsey, R., Nagel, P., Vigneau, D. and Sjoblad, R.: Skeletal responsiveness to parathyroid hormone in calciferol deficient rats. Clinical Research 21:633, 1973.

DETAILED BUDGET FOR FIRST 12-MONTH PERIOD

	Salary	Fringe*	TOTAL
Personnel			
Research Technician	$10,244	$1,229	$11,473
Research Technician	10,244	1,229	11,473
Supplies			5,470
^{40}Ca (^{48}Ca-free)			8,000
TOTAL DIRECT COSTS			$36,416
Indirect Costs (41.9% S & W effective 10/1/76)			8,584
		TOTAL REQUESTED	$45,000

BUDGET ESTIMATES FOR ALL YEARS OF SUPPORT

	Year 1	Year 2	Year 3
Personnel	$22,946	$26,158	$29,554
Supplies	5,470	5,743	6,030
^{40}Ca (^{48}Ca-free)	8,000	6,500	7,000
TOTAL DIRECT COSTS	$36,416	$38,401	$42,584
Indirect Costs	8,584	9,614	10,768
TOTAL COSTS	$45,000	$48,015	$53,352

*Fringe benefits for 1977 = 12%
1978 = 14%
1979 = 15%

BUDGET JUSTIFICATION

One research technician will be occupied 50—70% of the time with the ^{48}Ca and the ^{47}Ca determinations. The second technician will spend 70% of the time on the $1,25(OH)_2$ vitamin D_3 and parathyroid hormone assays. The remaining time, representing on the average 30—40% of each technician's time, will be devoted to the numerous assays for Ca, ionized Ca, P, Mg, Cr, glycosylated hydroxylysine, hydroxyproline, etc. The specimen aliquoting and ashing, and the support for metabolic beds and clinical care of the subjects, are provided by other funds, as are Dr. Neer's salary and the salaries of his associates in the Endocrine Unit.

The large expense for isotopes is due to the high cost of 48-calcium-free ^{40}Ca. A twenty day study with a daily monoisotopic calcium intake of 400 mg would require 8 grams of ^{48}Ca-free calcium. At the current price of $250 per gram this costs $2000. We have asked for a total of 11 such studies. To avoid wasting isotopes of this value on inconsequential experiments the approximate hPTH(1—34) dose range of interest for demonstrating skeletal effects is now being defined independently beforehand using renal and intestinal responses to the hormone.

I. RATIONALE

Detailed investigation during the Apollo and Skylab flights indicated that space flight produced a characteristic pattern of calcium loss in the astronauts, with an increase in urinary calcium excretion and a negative calcium balance. The changes began within days and persisted for the duration of the flight. There was also a negative balance for phosphorus, sodium, and magnesium. These losses presumably reflect a reduction in the amount of bone mineral, since 99% of the body calcium is in bone and since the loss of the other minerals could not be accounted for by a reduction in soft tissue weight. In fact, there was a suggestion of reduced bone mass in the ankle after the prolonged skylab flight.

Prolonged loss of bone of this degree will eventually predispose astronauts to fractures. In addition, the hypercalciuria predisposes them to the development of kidney stones during space flight. Unless solved, these problems may limit the allowable duration of space flight, limit the number of space shuttle flights for a given individual, and may determine the feasibility or infeasibility of inter-planetary travel by man.

The causes for these changes in calcium and bone mineral metabolism are not clear and probably multiple. Studies to date have placed major emphasis on the causative role of weightlessness with resultant loss of mechanical stresses on the skeleton. An extended series of experiments have been and still are being carried out on the development and prevention of bone mineral loss produced by bed rest, which serves as a model for the effects of decreased mechanical stress on bone. These experiments have focused particularly on evaluation of different mechanical shocks or exercises to restore mechanical stresses to the skeleton, or the use of drugs which prevent bone resorption (since bone resorption is increased during bed rest).

Loss of mechanical stress, though important, is almost certainly not the sole cause of bone mineral losses and calcium losses in space, and may not be the most important cause. Daily mechanical stresses and exercises during the skylab flight failed to prevent calcium and bone mineral losses, even though they maintained or improved physical conditioning of the astronauts. An equally important, or even more important, cause of bone and calcium losses in space may be glucocorticoid excess. Space flight increased the astronauts' urinary free cortisol excretion 1.5— 2 times, which probably reflects a 1.5— 2-fold increase in their mean level of plasma free cortisol. Increases in mean plasma cortisol were not tested since multiple blood samples throughout the day were not drawn, but a sustained increase in mean plasma free cortisol seems plausible, since it occurs during periods of physical or emotional stress (1,2). A two-fold increase in renal clearance of free cortisol (with mean plasma free cortisol remaining constant due to homeostasis) is a less likely explanation for the observed changes in urinary free cortisol excretion during space flight, since such enormous changes in renal cortisol clearance are unknown. A 1.5— 2 times increase in mean free cortisol in blood would increase urinary calcium excretion, cause a negative calcium, phosphorus, and magnesium balance, and lead to a decrease in bone mass. This is well attested by extensive clinical experience with mild glucocorticoid excess — Cushing's syndrome (3,4).

Space flight is also accompanied by an increase in renal sodium clearance despite a 1— 3 kg decrease in body fluid and normal or increased amounts of aldosterone and cortisol in the plasma. This combination of findings suggests

a marked increase in sodium clearance by the proximal tubule of the kidney, analogous to effects produced by "third factor(s)". At least part of the hypercalciuria is probably related to this phenomenon, since proximal tubular calcium clearance and sodium clearance are related, i.e., they increase and decrease proportionately under the influence of sodium loading and restriction, or a water or osmotic diuresis.

We therefore believe NASA, while continuing its long-standing interest in bed rest as a model for calcium losses during space flight, should shift its emphasis to include mild glucocorticoid excess and renal "third factor(s)" as additional important or even dominant causes of calcium loss in space. The proposals in section III of this application are related to research in the area of glucocorticoid excess, where some exciting recent developments are of interest. NASA-supported research on renal sodium clearance by third factor(s) during weightlessness or immersion now going on elsewhere, could easily be extended to include studies of renal calcium clearance, in line with the second suggestion. We therefore do not propose these latter studies ourselves, but think they should be done elsewhere.

The mechanisms outlined above can be investigated in earth-based models, and hypotheses and preventive measures developed. Whatever the hypothesis or the preventive, it ultimately must be tested in space in man. The methods which have so far been useful to investigate bone metabolism and calcium turnover in space in man are limited to measurements of urinary calcium, phosphorus and hydroxyproline losses, and measurements of calcium, phosphorus, magnesium, and nitrogen balance. There is a pressing need for more sensitive and more informative measurements of bone mineral turnover. Metabolic balance studies in addition to being excessively demanding, provide information only on the ratio of input to output of calcium, phosphorus, etc., and not the absolute level of either. Furthermore, they are not very sensitive to small changes in input or output of mineral from the skeleton, due to collection errors. Isotopic tracers have long been used to circumvent these problems in animal studies: the skeleton is prelabeled with ^{45}Ca, ^{47}Ca, or ^{32}P, and hormonal or other physiological manipulations imposed subsequently. The loss of skeletal isotope in urine and feces and the change in isotope specific activity in blood are then very sensitive indices of skeletal resorption. Excessive radiation burdens have prevented the extension of this methodology to man. The availability of stable calcium isotopic tracers ("heavy calcium") makes it technically possible to circumvent this limitation, and the practical feasibility of such an approach has recently been demonstrated in a small number of human subjects (5). We wish now to develop further this technique, demonstrate its sensitivity in comparision with other methods, and prove that it is the most sensitive method available for measuring small changes in skeletal calcium resorption in man. Such validation would establish this method as a suitable technique for subsequent studies in man during space flight. For this purpose, we have developed over the past 18 months, a high precision method for the measurement of small amounts of stable ^{48}Ca in biological fluids. As a convenient test system, we wish to evaluate the skeletal response to small physiological doses of infused human parathyroid hormone in normal volunteers. These experiments are described in section II.

II. STUDIES OF CALCIUM METABOLISM IN MAN USING STABLE CALCIUM ISOTOPES

A. Background

1. ^{48}Ca Assays

The use of stable isotopes of calcium in clinical investigation has been proposed intermittently over the past ten years. In 1965, McPherson (6) discussed the theoretical advantages of stable isotopes and reported the results of pilot studies on the use of ^{46}Ca and ^{48}Ca. To date, the only substantive reports involving clinical investigations with stable isotopes have been those of Heaney and Skillman (7) in studies on pregnant women, and Hansen (5) in studies on the measurement of osteolysis in man.

Stable isotopes have been little used because most biologists lack familiarity with the techniques and the equipment necessary for neutron activation analysis or mass spectrometry, and because ^{47}Ca is readily available and easy to use. We think that techniques involving stable isotopes deserve further development. There is increasing concern about the long-term effects of radiation, and the restrictions on radiation doses — especially in volunteer research subjects — have become progressively stringent. Minimizing radiation exposure for research studies is a particular concern in the space program, because of the exposure already imposed by space flight itself. Stable isotopes provide radiation-free markers that can be used repeatedly in normal volunteers and even in children and pregnant women.

In collaboration with the Nuclear Engineering Department at MIT, and the Rhode Island Nuclear Science Center, we have recently developed an accurate, high precision assay for calcium-48 in biological samples, and have used the double isotope method (^{47}Ca and ^{48}Ca) to measure intestinal calcium absorption in hypoparathyroid patients treated with 1-hydroxylated vitamin D. We now wish to use this method, and the method of Hansen *et al.* (5), to measure parathyroid hormone effects on bone calcium release in man.

To assay ^{48}Ca, blood or acidified urine is adjusted to pH 4.0— 5.6 and ammonium oxalate added to precipitate all calcium. The precipitate is washed, redissolved in nitric acid, and precipitated again with ammonium hydroxide. After washing, the precipitate, now nearly free of interfering sodium and chlorine, is dissolved in nitric acid containing a fixed amount of praseodymium (as an internal standard), and sealed into polyethylene tubes. Three tubes (usually two unknowns and a standard) are taped together and placed in a container for irradiation in the atomic reactor at MIT or at RINSC. Following a 15-minute irradiation, 200 microliters of the solution are removed from the sealed polyethylene tubes, placed in a counting vial, and the ^{49}Ca and ^{143}Pr measured with a GeLi detector and Canberra multi-channel analyzer. This detector eliminates entirely any interference from Na, Cl, Ar, Mn, or other elements. After correction for variations in calcium content (atomic absorption spectroscopy) and variations in neutron flux (praseodymium counts), one can calculate the calcium-48 content for the sample. Comparison of results in standards and samples provides the percent increase in calcium-48 content in the samples.

As a test of our procedure, a urine sample was divided into four equal aliquots. To three of these, calcium-48 was added in increasing amounts to provide a calcium-48 enrichment 20%, 40% and 60% above background. The resulting calibration curve (expected versus observed calcium-48 enrichment) was linear

with slope 1.06 ± 0.02 and an intercept indistinguishable from zero. As a check on reproducibility, unenriched urines have been analyzed repeatedly on the same or different days. The coefficient of variation is 1.2% with no deviation from week to week or from urine to urine. Finally, as a check on absolute accuracy, when multiple unenriched urines are compared to each other and to analytical grade $CaCO_3$, the ratio of ^{48}Ca content/total calcium content is constant.

We have used this ^{48}Ca assay in measurement of intestinal calcium absorption in man by the double isotope method: two milligrams of ^{48}Ca was administered intravenously and 5 microcuries of ^{47}Ca given simultaneously by mouth with 200 mg of carrier calcium as the chloride. The subsequent ratio of $^{47}Ca/^{48}Ca$ in urine or blood was used to measure the efficiency of intestinal calcium absorption:

Results of $^{47}Ca/^{48}Ca$ Absorption Test in Three Hypoparathyroid Patients On a Constant Diet

	% Ca Absorption	
	Before	After 5 days of Rx with 1α-(OH)D_3
Cumulative $^{47}Ca/^{48}Ca$ isotopic ratios in urine after oral administration of ^{47}Ca and intravenous ^{48}Ca.	15%	45%
	13%	57%
	26%	23%

The first two patients also had significant increases in serum and urine calcium. The third patient, who received a sub-therapeutic dose, did not.

The estimated radiation dose was 0.1 rads to gut and 0.08 rads to bone. Similar studies using radioactive ^{45}Ca as the intravenous isotope deliver an estimated 0.52 rads to gut and 1.4 rads to bone.

2. Clinical Methods of Measuring Calcium Release from Bone

Conventional calcium kinetic studies involve the intravenous administration of ^{47}Ca or ^{45}Ca and the subsequent determination of its disappearance curve in plasma. By measuring the specific activity curve, and fraction of the isotope excreted in urine and feces, one can determine simultaneously the rate constants and the absolute flux (mg/day) for calcium clearance by the skeleton, intestines, and kidneys (8). The rate of calcium re-entry to blood from the skeleton is then calculated indirectly (release = uptake - balance).

Calcium release from the skeleton can also be measured directly, using an ingenious method recently described by Hansen et al. (5). In this method, ^{48}Ca-free calcium is substituted for ordinary calcium in the diet. On such a diet, any ^{48}Ca entering the plasma must come from bone, i.e., bone is labeled with this naturally occurring isotope. During the first 7 to 10 days' equilibration on this diet, the $^{48}Ca/^{40}Ca$ ratio in plasma or urine declines asymptotically at a rate depending on the relative magnitude of dietary ^{40}Ca absorption and skeletal ^{48}Ca release. Thereafter any increase in the ratio of $^{48}Ca/^{40}Ca$ represents increased release of calcium from bone. These events are described by the formula:

$$E = \frac{Vo-}{Vt}$$

where E is the fractional ^{48}Ca abundance relative to ^{40}Ca

Vo- represents release of calcium from bone in mg/day
Vt represents turnover in the rapidly exchangeable pool (plasma)

E is determined by neutron activation analysis of ^{48}Ca in blood and Vt can be determined if total intake and excretion (fecal and urine) of calcium are measured; thus, the above formula can be solved for Vo-, release of calcium from bone.

Simultaneous measurements of calcium release from the skeleton by this direct method and by indirect methods (^{47}Ca kinetic studies vs ^{48}Ca-free diet studies) agree very well, but the direct method is more precise with uncertainties of $\pm$ 5% rather than $\pm$ 15% (5). The direct method using ^{48}Ca-free diets should therefore be far more sensitive to induced changes in the rate of bone calcium release.

3. Clinical Methods of Measuring Bone Matrix Release

Since the principal component of bone matrix is collagen, bone matrix resorption is conventionally assessed by measuring total urinary hydroxyproline excretion. This is a relatively insensitive and non-specific index in subjects with normal bone turnover, because non-skeletal collagen, dietary collagen, and non-collagenous proteins normally contribute significantly to urinary hydroxyproline. These extra-skeletal sources of urinary hydroxyproline become quantitatively insignificant only in individuals with very high rates of bone turnover (9). Furthermore, most of the hydroxyproline in collagen is catabolized; less than a third appears in the urine (10).

Measurements of urinary glucosylgalactosyl hydroxylysine (GGH) and galactosylhydroxylysine (GH) overcome these limitations entirely or partially. Excretion of collagen peptides containing these residues is nearly complete, and is unaffected by diet. Furthermore, the ratio of GGH/GH differs in different collagens, being significantly lower in bone collagen than in other collagens. Consequently, increased breakdown of skeletal collagen lowers the ratio of urinary GGH/GH while increasing total urinary hydroxylysine excretion (11). In contrast, increased breakdown of skin collagen or other collagens fails to lower the GGH/GH ratio in urine, despite comparable increases in total urinary hydroxylysine excretion (11,12). Combined measurements of urinary GH/GGH ratio and total urinary hydroxylysine thus offer a more specific and more sensitive index of bone collagen metabolism than measurements of urinary hydroxyproline (11,12,13,14 Their sensitivity has not, however, been directly compared with isotopic measurements of bone mineral turnover.

4. Skeletal Effects of Parathyroid Hormone

In dogs and rats, the initial (almost immediate) response to intravenous PTH administration is mild hypocalcemia (15). Calcium is taken up by the skeleton and perhaps acts as a second messenger for the later hypercalcemic response. Indeed, administration of small amounts of calcium (up to 8 minutes before the PTH) potentiates the later hypercalcemia (16). Subsequently, calcium and phosphate are released from bone under stimulation of PTH, though not

necessarily pari passu (17). Calcium release occurs at at least two different rates, reflecting pools with different degrees of calcium availability. Actinomycin D blunts the prolonged hypercalcemic response in rats but not the initial rise (18). The mechanism for this rapid initial mobilization of bone calcium is not known. The prolonged hypercalcemia and its delayed regression after PTH withdrawal do follow changes in cellular protein synthesis and multiplication. Rises in urinary hydroxyproline occur after PTH administration (19); however, this response lags behind the initial hypercalcemia. This difference may be an artefact due to different sensitivity of these two techniques for measuring skeletal resorption.

Bone cellular responses to PTH are complex. Barnicot (20) first described stimulation of osteoclasts in bone chips. Bingham et al. (21), using quantitative techniques in rabbits, found increased numbers of osteoclasts 22 to 26 hours after injection of parathyroid extract. Osteocytes also respond to PTH, but the quantitative significance of this response is unclear. Intracellular DNA and RNA synthesis, adenylate cyclase activity, and various enzyme activities and release are all promoted by PTH in bone cells (22,23,24). Collagenase and many of the lysosomal enzymes are secreted locally and presumably mediate bone calcium and matrix catabolism and release. Alkaline phosphatase is also secreted and this is associated with new bone formation.

Most attention has been paid to the catabolic bone response, but there is evidence for an anabolic action of PTH on bone. It has been shown repeatedly, since the early experiments of Bauer and Albright (25), that chronic parathyroid hormone injections increase bone density and bone mass in rats (25— 31). Kalu et al. (31) recently confirmed this observation with purer hormone and demonstrated that it was not a response to compensatory increases in calcitonin. Labeled amino-acid incorporation into collagen is increased by PTH injections in animals, and biopsy techniques show increased formation of new matrix and bone in such animals (31). There are numerous reports of osteosclerosis occurring in growing children with hyperparathyroidism, and recently Genant et al. (32) reported diffuse osteosclerosis in a 53-year-old woman with surgically-proven hyperparathyroidism and no other apparent cause for osteoclerosis. Calcium kinetic studies and bone morphometric studies invariably show increased bone turnover in primary hyperparathyroidism (33,34,35). Both anabolism and catabolism are increased. Catabolism predominates and osteopenia results if the hyperparathyroidism is severe (the higher the serum calcium, the more negative the calcium balance) (36). In mild hyperparathyroidism, with serum calcium below 12 mg%, calcium balance is seldom negative. In fact, total body retention of orally administered calcium-47 was on the average increased by hyperparathyroidism in two different studies (37,38).

The relative predominance of skeletal catabolism or anabolism seems to depend primarily on the dose of PTH in animal studies, though other variables have not been systematically studied. Separation of these two effects, if possible, would be of therapeutic interest in many metabolic bone diseases including osteoporosis. Most human and animal experiments on the effects of PTH on bone involve large doses of PTH, parathyroid extract, or the pathologic state of hyperparathyroidism. It is of current interest to determine whether anabolic effects predominate at smaller dose levels of PTH in man, or are dependent on the duration of exposure to PTH. Quantitative data to answer this question in man are nearly non-existent (36,39).

The role of PTH in the pathogenesis of osteoporosis in man is unclear. Idiopathic osteoporosis probably has several pathophysiologic bases. Riggs et al. (40) identified some osteoporotic patients with PTH excess, but the majority had normal or undetectable blood levels of immunoreactive PTH. Harris and Heaney (41) suggested that new bone formation might be reduced in patients with low PTH allowing resorption to occur at a slow but unopposed rate. Reeve et al. have recently given daily subcutaneous injections of parathyroid hormone to four osteoporotic patients in Great Britain for periods of 6—18 months (42,43). Increases in intestinal calcium absorption occurred in all. Improvements in calcium balance occurred with doses of 200 micrograms/day, but not with higher doses in two patients. No changes in renal or hepatic function or complete blood count occurred during chronic therapy, and daily hormone injections produced neither local nor systemic side effects, nor were any antibodies detectable in the serum of the chronically-treated patients.

5. Synthetic Preparations of Human Parathyroid Hormone

Two groups have reported the sequence of the first 37 amino acids of human parathyroid hormone. Both groups agree on the sequence of the first 21 amino acids, and it is notable that the sequence is identical to that of bovine PTH except position 16, where asparagine is substituted for serine. Brewer et al. (44) have found glutamine at position 22, lysine at 28 and leucine at 30. Niall et al. (45) have shown these positions to be glutamic acid, leucine and aspartic acid. Reinvestigation of this sequence by Niall and colleagues has confirmed their original findings (46).

The amino-terminal segment of parathyroid hormone is necessary and sufficient for biological activity as defined by bioassay techniques. This has been established for both porcine and bovine PTH, and the critical fragment length appears to include an intact peptide sequence of 26 amino acids from the amino terminus of the molecule PTH(2—27) (47,48). PTH(1—34) has a potency of 77% of intact hormone in the rat renal cortical adenylate cyclase assay, and 132% in the chick hypercalcemia assay. A peptide consisting of the amino-terminal 34 acids of the human sequence has been synthesized and is available in sufficient quantity to be used for clinical investigation (49). The biologic activity of hPTH(1—34) (Niall sequence), as measured by the rat renal cortical adenylate cyclase assay, is identical with the 1—84 native hormone on a molar basis and is of comparable potency to bovine 1—84 native hormone in the chick hypercalcemia assay. (Sufficient quantity of hPTH, native hormone, is not available for evaluation in the latter assay). In contrast, hPTH (Brewer sequence) has a much lower biological activity in both assays. Radioimmunoassay with five different antisera gives reacitons of identity with hPTH(1—34) (Niall) and native hPTH. hPTH(1—34) (Niall) is approved for human use in patients and normal volunteers in our institution and we have recently received an IND from the Food and Drug Administration covering this usage. In view of our inability to confirm the Brewer sequence, our re-affirmation of the Niall sequence, the anomalously low biological activity of the Brewer sequence synthetic hPTH(1—34) (when compared to either native human PTH(1—84) or Niall sequence synthetic peptide) and the non-antigenicity of Niall hPTH(1—34) in man, we believe the Niall sequence is correct and the proposed studies appropriate.

B. Specific Aims

a. Develop further the use of stable calcium isotopes to measure small changes in bone resorption in man, and demonstrate their sensitivity in relation to other available measures of bone resorption.

b. Determine the lowest dose of parathyroid hormone that will exert skeletal

effects in vivo on bone in man, and thereby develop a quantitative description relating PTH entry rates to PTH effects on bone turnover in man.

C. Methods of Procedure

The recent structural analyses of human parathyroid hormone carried out in this unit have permitted, for the first time, synthesis of human parathyroid hormone in quantities sufficient for clinical investigation. As a result, it is now possible to establish, in man, quantitative relationships between human parathyroid hormone infusion rates, and biological responses in bone. We wish to measure skeletal responses to graded doses of human parathyroid hormone, starting with homeopathic doses and increasing the dose by factors of two until a serum calcium above 10.5 mg% is produced. Such experiments are of considerable theoretical interest, since they will provide a quantitative description of the skeletal role in calcium and phosphorus homeostasis to complement current qualitative descriptions. They also provide a well-defined system in which one can measure small changes in skeletal resorption in response to physiological doses of a hormone. In this system we wish to compare the sensitivity of total balance studies for calcium and phosphorus, conventional ^{47}Ca kinetic studies, urinary hydroxyproline excretion, urinary GGH and GH, and measurements (5) of skeletal ^{48}Ca release as indices of bone resorption.

For these studies, normal volunteers will be admitted to our metabolic ward and given a 200 mg Ca, 1000 mg P, 100 meq Na, low gelatin constant diet, and 400 mg additional oral calcium as the chloride each 24 hours. For three weeks prior to admission and throughout the study, volunteers will take daily capsules containing 150 mg chromium as the sesquioxide. After six days equilibration to the metabolic routine, ^{48}Ca-free calcium chloride will be substituted for the natural calcium chloride, and 10 microcuries of radioactive ^{47}Ca will be injected intravenously. Multiple blood samples would be taken that day, from an indwelling needle, to measure ^{47}Ca disappearance rates. For the next 20 days, 24 hour urine samples will be collected daily to determine ^{47}Ca, ^{48}Ca, and total Ca. During the last 10 of the 20 days, hPTH(1—34) will be given by constant intravenous infusion through a portable syringe pump, at one of two doses. During these days, a blood sample will be drawn every 12—24 hours to measure calcium and phosphorus concentrations.

In these studies the use of ^{48}Ca-free calcium and a very low calcium diet makes the skeleton the only significant source of ^{48}Ca entering the plasma. As a result, the ratio of ^{48}Ca/total Ca in blood or urine falls progressively. After 10 days, the rate of decline in the ratio of ^{48}Ca to total Ca is relatively slow. A sudden increase in the ratio of ^{48}Ca to total Ca will then signal an increase in skeletal Ca release, and the magnitude of skeletal calcium resorption can be calculated. For these studies, hPTH infusions will be given for 5—10 days, a period we calculate to be more than sufficient to increase the $^{48}Ca/^{40}Ca$ ratio in blood if bone resorption of calcium is significantly enhanced. Since the precision of basal measurements with this method is $\pm$ 30—60 mg/day, if we increase skeletal release of calcium by 100 mg daily for 10 days, we will clearly be able to measure the change in ^{48}Ca release. Calcium balance methods can detect changes of this magnitude on a normal diet only with difficulty.

Simultaneously, all feces will be collected in 5 day pools for analysis of ^{47}Ca, total Ca, phosphorus and chromium. When combined with repeated measurements of dietary calcium and phosphorus content, this provides the necessary

additional data for a conventional analysis of ^{47}Ca kinetics, total calcium balance and total phosphorus balance. For the latter analyses, fecal excretions will be corrected for variations in intestinal emptying by using the percentage recovery of the orally administered non-absorbable chromium marker. Finally, the daily urine collections will be analyzed for total hydroxyproline, total hydroxylysine, GGH and GH. The analytical methods to be used in these studies are described in section IV.

During these studies the infusion site and catheter will be switched every 48 hours and an iodine skin prep used to minimize the possibility of systemic infection. Antibiotic ointment will be applied daily to the entry site, and oral temperature measured four times daily. With these precautions, we believe there is no hazard from these studies, since pure human parathyroid is used. The doses are not sufficient to cause hypercalcemia, and the doses of radioactivity are well within acceptable limits. The studies have been approved by the Hospital's Committee on Human Studies, its Pharmacy Committee, and its Radioactive Isotope Committee (as have all the other studies in this application).

III. PATHOPHYSIOLOGY AND PREVENTION OF GLUCOCORTICOID-INDUCED BONE LOSS

A. Background

1. Clinical Experience

Glucocorticoid osteoporosis is an insidious process affecting all bones. The osteoporosis frequently becomes clinically apparent first as a fracture or collapse of the vertebral bodies. Fractured hips, wrists and long bones are also common. It is difficult to estimate precisely the incidence of osteoporosis due to glucocorticoid excess since there are multiple variables which will produce individual variations, such as the degree of glucocorticoid excess, the duration, the patient's age, exercise, calcium and vitamin D intake, and underlying diseases. However, the frequency of osteoporosis can be estimated approximately from descriptions of natural glucocorticoid intoxication (Cushing's syndrome) and iatrogenic intoxication. Among the 43 unpublished cases of Cushing's syndrome studied by Fuller Albright's group at this institution, osteoporosis was one of the most common serious complications, being present in 39 patients (50). In 1942, Sussman and Copleman (51) presented the first summary of the skeletal changes in Cushing's syndrome, noting osteoporosis particularly in the axial skeleton. Various reviews have added further support to this association (52-55). Pathologic fractures, generally compression fractures of the vertebrae or ribs, were present in 32—43% of the 196 patients in these studies. In 1950, the first report of spontaneous fracture in patients on cortisone therapy for rheumatoid arthritis was published (56). Similar findings were soon reported in other patients receiving glucocorticoids for rheumatoid arthritis (57), lupus erythematosus (58), and pemphigus vulgaris (59). Reifenstein in 1956 (60) described 38 patients with spontaneous fractures complicating glucocorticoid therapy, and Murray in 1960 (61) confirmed these earlier reports. Attempting a controlled analysis, McConkey and co-workers (62) found 5 patients with vertebral crush fractures among 61 glucocorticoid-treated rheumatoid arthritics, and no fractures in 36 rheumatoid controls. There was no significant difference in the incidence of osteopenia (independently assessed) in the two groups. Gallagher et al. found vertebral crush fractures in 17/27 patients receiving glucocorticoids for various medical problems (63). The incidence of crush fractures cannot be obtained from these figures since many patients were referred specifically because of their crush fracture. However, two of the men were below age 50 and 3 women were below age 40, making it reasonably certain that there was a relationship between glucocorticoid Rx and the vertebral compression fractures.

Presently there is no treatment of proven effectiveness for glucocorticoid-induced bone disease. Elimination of the glucocorticoid excess halts the progress of the disease, but with the exception of a few patients affected before puberty, regrowth of bone does not occur (64,65).

2. Glucocorticoids and Bone Turnover

Quantitative morphological studies of bone biopsies suggest glucocorticoid excess decreases bone formation and increases bone resorption (66). Storey in 1961 (67) gave large doses of cortisone to rabbits and induced osteoporosis with large numbers of osteoclasts at trabecular margins. Gallagher and co-workers also found glucocorticoid intoxication increased bone-resorbing surfaces and possibly decreased the amount of bone-forming surfaces (63). Riggs, Jowsey and Kelly (68), using quantitative micro-radiography, studied 12 cases

of natural Cushing's syndrome and found decreased bone-forming surfaces in all and increased bone-resorbing surfaces in 7. It has also been shown that glucocorticoids reduce collagen synthesis in bone (69,70), as estimated by radioactive amino-acid incorporation studies. In summary, glucocorticoid excess seems to increase bone resorption and decrease bone formation, but the relative importance of each is uncertain and may depend on experimental techniques or other variables (see below).

3. Glucocorticoids, Parathyroid Hormone, Calcitonin and Bone Mineral Homeostasis

The abnormalities in bone formation and resorption are associated with increased urinary calcium excretion, decreased intestinal calcium absorption, and negative calcium balance. It is not clear whether these abnormalities are direct effects of glucocorticoids, or homeostatic adaptations to glucocorticoid excess. Central to this controversy is the role played by parathyroid hormone (PTH). Glucocorticoids decrease the serum calcium concentration in animals if compensatory changes in parathyroid function are prevented by prior parathyroidectomy (71). In intact animals, no fall in serum calcium concentration occurs (71), presumably because of compensatory hyperparathyroidism. These results suggest that glucocorticoids in large doses provoke secondary hyperparathyroidism, and Kukreja and co-workers have reported that glucocorticoids do increase parathyroid hormone secretion (72). However, the same workers also report that parathyroidectomy did not protect cortisone-treated animals from developing osteoporosis (73). These animal experiments, though very interesting, may not be applicable to the problems of space because of the massive doses of cortisone used (5 mg/kg/day). However, the same workers have also reported that intravenous infusions of hydrocortisone in therapeutic doses immediately increase serum PTH levels in normal humans (74). They also found increased levels of immunoreactive PTH in patients chronically treated with glucocorticoids (74).

Direct inhibitory effects of glucocorticoids on parathyroid hormone secretion have been claimed by Eliel and co-workers (75,76). However, Talmage et al. (77,78) found that giving cortisol to rats in very large doses (5 mg/kg/day) for five days did not alter the ability of their parathyroid glands to respond to the challenge of calcium deficiency.

Stimulated by these reports, we have recently completed an analysis of immunoreactive PTH levels in over 50 glucocorticoid-treated asthmatics, and are unable to confirm the presence of hyperparathyroidism. PTH levels were normal (as they are during space flight). These discrepant results may reflect differences in PTH immuno-assay specificity, differences in glucocorticoid dosages, or disease-related differences in PTH excretion and metabolism in different patient groups. At any rate, it is by no means clear that PTH secretion is altered in humans by chronic glucocorticoid excess.

More consistent results have been obtained in studies of PTH-cortisol antagonism in PTH target organs. Eliel et al. found cortisol antagonizes the effects of PTH on bone and kidney (75,76). Jee et al. (79), using tritiated thymidine labeling of precursor cells of alveolar bone, found that the effects of cortisol on this target organ were dose-dependent. Low dose levels of cortisol stimulated precursor cell proliferation, increased the number of osteoclasts, and increased bone resorption, and decreased bone formation. These effects are identical to those of parathyroid hormone. At high cortisol doses, however, there was depressed precursor cell proliferation and decreased bone formation without any evidence of bone resorption. Talmage et al. (77,78)

demonstrated that adrenalectomy facilitated the removal of radioactive calcium which had been in bone for 2 or more weeks, whereas cortisol interfered with its removal. Since removal of this "deep" labeled bone is known to be PTH-dependent, these results suggest a possible cortisol-PTH antagonism. The relative importance of these various interactions between glucocorticoids and PTH, and the dose-dependence of the various interactions, require further study. Furthermore, some of these interactions need to be reevaluated using pure glucocorticoids without mineralocorticoid-like actions.

Thyrocalcitonin (CT) produces hypocalcemia and hypophosphatemia, largely due to an inhibition of bone resorption (80), though there are also increases in renal calcium and phosphorus clearance and bone phosphorus uptake (81,82). Aldred et al. (83) reported that adrenalectomy had no effect on the hypocalcemic response to CT in rats. These results were confirmed by Thompson and co-workers (84). However, they also found that in rats given pharmacologic doses of cortisone, the hypocalcemic effect of CT was significantly inhibited, whereas the hypophosphatemic effect was not altered (84,85). Calcitonin secretion in the rat seems to be unaffected by cortisol treatment, at least in doses of 5 mg/kg/day for five days (77,78). Because calcitonin inhibits bone resorption, whereas glucocorticoids have the opposite effect, Thompson et al. evaluated the therapeutic potential of exogenously administered porcine calcitonin in the treatment of glucocorticoid-induced osteoporosis. Using rabbits (86) they were able to show in those with cortisone-induced bone changes that CT slowed cancellous bone resorption, increased the rate of bone formation in the femoral diaphyses, increased the mass per unit area in cortical and cancellous bone, decreased the radiographic rarefaction of femora and vertebral bodies, and increased the ash content of vertebral bodies. These results are very important. The findings prompted them to study the effects of calcitonin on a 65-year old woman with severe osteoporosis who was on long term prednisone therapy for chronic lymphocytic leukemia and who presented with a compression fracture and generalized osteopenia (87). Clinically she improved during calcitonin treatment, and her urinary calcium and hydroxyproline decreased. However, during the same interval the prednisone dose was decreased, so one cannot be certain that the improvement was due to calcitonin. These findings deserve further clinical investigation.

4. Glucocorticoids, Vitamin D, and Intestinal Calcium Absorption

Glucocorticoids dramatically lower serum calcium levels in situations characterized by vitamin D overdosage (88) and/or hypersensitivity (89). They also interfere with the vitamin D treatment of hypoparathyroidism (90). This antagonism was also demonstrated by Harrison and Harrison (91) who showed that cortisol treatment reduced the rate of vitamin D-mediated intestinal calcium absorption, using the in vitro everted gut-sac technique in rats. Williams and co-workers and Kimberg and co-workers have confirmed these results and extended them to other glucocorticoids, using very large doses (92,93).

Avioli (94) reported that glucocorticoids change radioactive vitamin D kinetics in man, shunting radioactivity away from 25-OHD$_3$ and toward biologically inactive metabolites. These kinetic studies are difficult to interpret, because they describe only the turnover of [^{3}H]D$_3$ without correction for its absolute concentration in plasma. Differences in vitamin D$_3$ or 25-OHD$_3$ pool size therefore could not be taken into account, and absolute vitamin D$_3$ or 25-OHD$_3$ disposal rates were unknown. Kimberg et al. found no abnormality in radioactive vitamin D metabolism in extensive studies in rats with an intestinal calcium absorptive defect caused by glucocorticoids (93). Furthermore, the defect in calcium absorption in these rats was not corrected by massive doses

of D_3 or modest doses of 25-OHD_3 (93). Favus, Kimberg and co-workers (95) also found that glucocorticoids do not interfere with conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ or with the cellular and subcellular localization of this metabolite in the intestines. However, their chromatographic methods are now known to be inadequate for the separation of 1,25-$(OH)_2D_3$ from other dihydroxy metabolites. The same is true of the report of Lukert and co-workers (96), who found in prednisolone-treated rats an increased concentration of "1,25-$(OH)_2D_3$" in serum and intestinal mucosa, coincident with a reduced intestinal transport of calcium.

Of considerable interest is the discovery by Favus et al. (97) that 1,25-$(OH)_2D_3$ in physiologic doses overcame 50% of the cortisone-induced defects in intestinal calcium transport, as measured with modified Ussing chambers. Carré and co-workers (98) found that glucocorticoids stimulate the conversion of 1,25-$(OH)_2D_3$ to a more polar, biologically inactive metabolite in the intestine. In contrast, nearly normal amounts of 1,25-$(OH)_2D_3$ were found in liver, kidney and bone extracts obtained from glucocorticoid-treated animals. They also found that when calcium transport in isolated intestinal loops was measured 7 hours after 1,25-$(OH)_2D_3$ dosage, there was a marked increase in both control vitamin D-deficient rats and prednisolone-treated vitamin D-deficient rats. However, when measured at 24 and 48 hours after the 1,25-$(OH)_2D_3$ was given, the response was significantly less in prednisolone-treated animals. Favus et al. (97) made measurements at 16 hours after 1,25-$(OH)_2D_3$ dosage. It thus appears that the effects of 1,25-$(OH)_2D_3$ on intestinal calcium transport are abbreviated by glucocorticoid therapy, due to alterations in intestinal metabolism of 1,25-$(OH)_2D_3$.

The relevance of these duodenal abnormalities to glucocorticoid osteoporosis is unclear. Experiments in our laboratory have shown only trivial effects of glucocorticoids on total intestinal absorption of calcium-47 in the rat. Neither severe dietary calcium deprivation nor partial vitamin D deficiency altered this finding. These results confirm earlier calcium balance studies in rats (99) and are consistent also with the fact that glucocorticoids inhibit active transport of calcium only in the rat duodenum, not in the jejunum or ileum (100). Since overall intestinal calcium absorption is nearly normal in glucocorticoid-treated rats, and their bone levels of 1,25-$(OH)_2D_3$ are nearly normal (98), their osteoporosis may have little relation to the demonstrated alterations in duodenal calcium transport and duodenal 1,25-$(OH)_2D_3$ levels.

There is a surprising lack of information on intestinal calcium absorption and blood vitamin D levels in glucocorticoid-treated patients, except for the study by Caniggia and Gennari (101). They found most patients on short term glucocorticoid therapy had impaired intestinal calcium absorption, which could be counteracted by simultaneous treatment with pharmacologic doses of vitamin D_3. In those patients who received glucocorticoid therapy for one year or more, calcium absorption was not further worsened by the additional treatment period. However, pharmacologic doses of vitamin D_3 did not improve the impaired absorption of calcium in these chronically treated patients. 25-OHD_3 (0.1 mg/day for 20 days) in three patients did result in an appreciable improvement of intestinal calcium transport. This discrepancy between long- and short-term treated patients is unexplained. Furthermore, the above report failed to demonstrate consistent effects on calcium absorption even in short term studies.

We have recently begun a longitudinal survey of glucocorticoid-treated asthmatics, monitoring blood 25-OHD and PTH levels, and correlating them with serial changes in bone mass and blood calcium concentrations. After initial evaluation, many of the patients are being switched from prednisone to a non-absorbable inhaled glucocorticoid. The remainder are continuing to take oral prednisone. The results of the initial evaluations are summarized as follows:

Prednisone oral dose	PTH	25-OHD
< 10 mg/day	↑ 0/19	↓ 4/17
10—19 mg/day	↑ 0/29	↓ 5/26
20—29 mg/day	↑ 2/12	↓ 0/12
≥ 30 mg/day	↑ 1/3	↓ 0/12
TOTAL PATIENTS	60	57
NORMAL RANGE	< 10	15-80

The significant incidence of low 25-OHD levels in these patients is unexplained, since blood vitamin D measurements are not available to exclude coincidental vitamin D deficiency. Furthermore, these asthmatics receive many drugs in addition to glucocorticoids. Whatever the reason, vitamin D supplements are clearly indicated in some of these patients. Our serial followup study allows us to monitor the effectiveness of such Rx in maintaining a normal blood 25-OHD level. Hahn et al. (102) have given vitamin D or 25-OHD in pharmacological doses to glucocorticoid-treated patients with rheumatoid arthritis and have found an increase in bone mass within 12 months. Whether these results suggest reversal of glucocorticoid effect or merely a cure of incidental vitamin D deficiency is unclear. Many unanswered questions remain, and serum estimation of vitamin D_3, $1,25\text{-}(OH)_2D_3$ and assessment of metabolic clearance rates and production rates of $1,25\text{-}(OH)_2D_3$ may add more information to this confused picture.

5. Glucocorticoids and Bone Mineral Excretion by the Kidney

Increased urinary excretion of calcium is a common finding in patients receiving glucocorticosteroids. Pechet and co-workers (103) found in three normal volunteers that prednisolone, 30 mg/day, had little effect on urinary calcium and phophorus. In a dose of 60—280 mg daily prednisolone increased urinary calcium and phosphorus excretion. Laake (104) performed calcium clearance studies in sixteen patients receiving long-term therapy with small doses of corticosteroids. He found increased tubular clearance in seven. Wajchenberg and co-workers (105) found that dexamethasone (12 mg/day) increased the renal clearance of diffusible calcium in a patient with idiopathic hypoparathyroidism, implying that the changes in renal tubular calcium reabsorption are not due to changes in PTH secretion. Caniggia and Gennari (101) found in patients receiving short-term glucocorticoid therapy a significant increase in urinary calcium excretion. Similar findings were present in 27 patients reported by Gallagher (63). The explanation for this increased excretion and increased renal clearance of calcium is not apparent from published data. It is known that sodium loading, or mineralocorticoid-induced extracellular fluid expansion, increases renal sodium and calcium clearance via changes in proximal tubular function (106,107). Furthermore, mineralocorticoid-induced increases in renal calcium clearance can be blocked by dietary sodium restriction in rats (108). An analogous phenomenon might underlie the increased renal calcium clearance generated by glucocorticoids, but this hypothesis has not been adequately tested. It is known that glucocorticoids have no immediate effect on renal calcium clearance in animals (109) and man (110), suggesting that indirect effects are operative.

Normally renal phosphate clearance increases progressively during the forenoon. Goldsmith and co-workers (111) suggested that variations in hydrocortisone secretion were responsible, for they found an increase in the excretion of filtered phosphate as the plasma hydrocortisone concentration declined, and they found no diurnal variation in Addisonian patients. In apparent contrast, Roberts and Pitts (112) reported that in the phosphate-loaded dog, acute hydrocortisone administration increased phosphate clearance and excretion. Others (113,114) have shown that the administration of glucocorticoids for 24 hours or longer results in increased phosphaturia, but the degree of phosphaturia at any time depends upon the balance among several hormones. Because of the variety of factors influencing renal phosphate clearance, the effect of glucocorticoids on this function has received relatively little attention.

Since renal calcium clearance and renal phosphate clearance are both increased by extra-cellular volume expansion, a particular glucocorticoid's effect on these functions will be strongly influenced by the degree of ECF expansion it produces. It therefore seems unrealistic to equate subacute or chronic studies with cortisone and subacute or chronic studies with pure glucocorticoids such as prednisone or dexamethasone, unless experimental data support their equivalence. This potential error infects much of the work on glucocorticoid-induced changes in renal function.

B. Specific Aims

1. Evaluate more fully parathyroid function in normal volunteers acutely treated with glucocorticoids and patients chronically treated with glucocorticoids.

2. Define the inter-relationship among 1,25-$(OH)_2D_3$ blood levels and intestinal calcium absorption in patients chronically treated with glucocorticoids and (if advisable) normal volunteers acutely treated with glucocorticoids.

3. Evaluate the therapeutic potential of 1,25-$(OH)_2D_3$ in patients chronically treated with glucocorticoids.

4. Evaluate the effects of sodium deprivation on renal losses of calcium and phosphorus produced by cortisone or prednisone.

C. Methods of Procedure

1. Evaluate More Fully Parathyroid Function in Normal Volunteers Acutely Treated with Glucocorticoids and Patients Chronically Treated with Glucocorticoids

The discrepancy between the elevated blood PTH levels found by Williams et al. (74) in glucocorticoid-treated patients, and the normal levels found by us, may be due to differences in glucocorticoid dose, underlying diseases, or assay techniques. Half of our patients took $\leq$ 10 mg prednisone/day and all took less than 30 mg/day, whereas those of Williams were receiving 15—80 mg daily. We are currently collecting data from patients chronically receiving larger doses of glucocorticoids and from patients receiving glucocorticoids for illnesses other than asthma. These samples will be assayed in our standard PTH radioimmunoassay, which is sensitive to both biologically active amino-terminal and biologically inactive carboxy-terminal PTH antigens. Parallel assays will also be run using an amino-terminal.

specific antiserum recently raised by immunization with synthetic hPTH(1–34). The combined studies should establish whether hyperparathyroidism in glucocorticoid-treated patients is dose-related, disease-related, or a technical artefact. In addition, six patients chronically treated with low doses of prednisone and six chronically treated with high doses will be admitted to the metabolic unit for an EDTA infusion (115), a procedure useful for demonstrating parathyroid hyperfunction in patients with mild hyperparathyroidism (116).

If, as we suspect, large doses of glucocorticoids induce hyperparathyroidism and smaller doses do not, it will be important to establish why such increases occur, i.e., whether PTH hypersecretion is appropriate (secondary hyperparathyroidism), or inappropriate (direct effect on PTH secretion/release) or both. If so indicated, normal volunteers will be admitted to the metabolic unit on a constant 200 mg Ca, 100 meq Na diet, and after equilibration and control periods, given prednisone in PTH-elevating doses. Serum PTH levels will be measured daily and correlated with serial changes in blood and urine calcium and phosphorus, blood ionized calcium, urinary hydroxyproline and glycosylated hydroxylysine, weight, and intestinal calcium absorption, using methods described in section IV below. If hyperparathyroidism is appropriate, it should be preceded or accompanied by increases in urinary calcium, decreases in intestinal calcium absorptive efficiency, or changes in serum ionized or total calcium. Changes in net skeletal calcium balance, although important as well, cannot be detected rapidly enough to establish such temporal relationships, and their causative role can only be evaluated by exclusion, or indirectly by changes in bone collagen degradation.

2. Define the Inter-relationships Among 1,25-$(OH)_2D_3$ Blood Levels and Intestinal Calcium Absorption in Patients Chronically Treated With Glucocorticoids and (if advisable) Normal Volunteers Acutely Treated with Glucocorticoids

Patients chronically recieving glucocorticoids will be admitted to the Metabolic Unit and given a 200 mg Ca, 800 mg P, 100 meq Na diet to assess their ability to adapt intestinal calcium absorption to a low calcium intake. During six days of dietary equilibration, a 3 day stool fat measurement and a D-xylose absorption test will be done to exclude generalized malabsorption. At the end of the equilibration period, intestinal calcium absorptive efficiency will be measured by double-isotope techniques. If calcium absorption is low (normal >50% on this diet), the 1,25-$(OH)_2D_3$ blood level will be measured using the methods outlined below (section IV). The patient will subsequently be given 1,25-$(OH)_2D_3$ (1 microgram) or 1α-OHD_3 (2 micrograms) or D_3 (500 micrograms) daily for three days, and calcium absorptive efficiency measured again. The results of all tests will be correlated with the simultaneous serum Ca, P, PTH, 25-OHD, alkaline phosphatase, 24-hour urine calcium, and forearm bone mass (photon densitometry of the radius). The 25-OHD blood levels will be necessary to exclude coincidental vitamin D deficiency (see above).

If these studies document abnormalities in calcium absorption, they will be repeated in normal volunteers given glucocorticoids, to determine the glucocorticoid dose or the duration of therapy needed to impair intestinal calcium absorption. At the same time, 1,25-$(OH)_2D_3$ blood levels will be studied in these subjects, to see whether such measurements can be used to predict intestinal calcium absorptive efficiency during glucocorticoid excess.

3. Evaluate the Therapeutic Potential of 1,25-$(OH)_2D_3$ in Patients Chronically Treated with Glucocorticoids

If significant impairments in intestinal calcium absorption are detected, which are corrected by vitamin D, 1,25-$(OH)_2D_3$ or 1α-OHD_3, it will be imperative to test the thesis of Boisseau, Hahn and Avioli (102) that a true increase in bone mineral density (and by implication a positive calcium balance) can be achieved by high dose vitamin D therapy of glucocorticoid-treated patients. A selected group of patients with significant osteopenia who are receiving glucocorticoids in relatively stable dosages and who have no evidence of renal insufficiency (normal creatinine clearance), will be evaluated in the metabolic unit as described above, and then discharged on chronic 1,25-$(OH)_2D_3$ or D_3 therapy in effective doses (determined as above), plus supplemental calcium if needed to insure a dietary calcium intake of 1500 mg/day. Patients will be readmitted at six-month intervals and evaluated by bone densitometry, repeat intestinal calcium absorption studies, serum calcium, phosphorus, alkaline phosphatase, PTH, 25-OHD and 24 hour urine calcium. Encouraging results will be a statistically significant increase in bone mineral density by photon absorption or other skeletal measurements. Further patients will be added if initial results are encouraging. Blood and urinary calcium will be monitored at least monthly in these patients, and therapy adjusted if hypercalciuria develops and stopped if hypercalcemia develops. A single iliac crest bone biopsy will be done before therapy. If osteomalacia is found a repeat biopsy will be performed after six months adequate 1,25-$(OH)_2D_3$ therapy.

4. Evaluate the Effects of Sodium Deprivation on Renal Losses of Calcium and Phosphorus Produced by Cortisone or Prednisone

In the course of the studies described above, patients and normal volunteers receiving pure glucocorticoids will be studied on the metabolic unit while consuming a 100 meq Na diet of constant Ca and P content (usually 1000 mg each). When their studies are complete, their dietary sodium will be decreased acutely to 10 meq daily, and urinary and plasma calcium and phosphorus, blood PTH, weight, BUN, creatinine, standing and supine blood pressure, and plasma renin measured daily for four—six days.

In addition, six normal volunteers will be admitted to the ward, and given a 100 meq Na, 1000 mg Ca, 1000 mg P diet. After equilibration and control studies, cortisone acetate 50 mg BID will be given until the urinary calcium increases and plateaus (approximately 5—8 days). Thereupon dietary sodium will be abruptly decreased and measurements continued as above. Similar studies will be carried out in selected patients with naturally occurring Cushing's syndrome. This comparison of the effects of cortisone with the effects of pure glucocorticoids in man seems necessary to gain insight into the calcium and bone losses of naturally-occurring glucocorticoid excess, and to allow more intelligent appraisal of experimental studies employing different glucocorticoids.

IV: ANALYTICAL METHODS

The following methods currently are in use in this Unit routinely:

Calcium, magnesium and chromium are analyzed by atomic absorption spectroscopy, inorganic phosphate by a modified Gomori method, hydroxyproline by the method of Kivirikko et al. (117), and calcium-47 is analyzed in plasma in a two inch NaI well crystal (Packard) and in urine and feces in 2500 ml Marinelli beakers with a heavily shielded three inch NaI detector (General Atomium). Urine creatinine is measured by the alkaline picrate method; plasma creatinine is similarly measured after initial removal of non-creatinine chromogens (118). Urine and blood pH and pCO_2 are measured with a Radiometer electrode system. Glycosylated hydroxylysines are measured by the method of Kelleher and Bisbee (119). Sodium and potassium are measured by flame photometry in the MGH clinical chemistry laboratory. Urine cyclic AMP is measured by the method of Gilman (120). Diets and feces for the balance studies are ashed twice in an electric furnace and dissolved in dilute HCl before analysis. Parathyroid hormone is measured by radio-immunoassay (121).

At present, Brumbaugh et al. (122) have developed the only assay of $1,25\text{-}(OH)_2D_3$ widely validated in human studies. This assay depends on the binding of $1,25\text{-}(OH)_2D_3$ to its chromatin receptor in the small intestine. The receptor is specific for $1,25\text{-}(OH)_2D_3$ and the radioactive hormone is displaced from its binding site only by much higher concentrations of $25\text{-}(OH)_2D_3$ (150-fold excess) and vitamin D (20,000-fold) excess (122). To determine the concentration of $1,25(OH)_2D_3$ in human plasma, after lipid extraction with methanol and chloroform, the $1,25\text{-}(OH)_2D_3$ is isolated from other vitamin D metabolites on LH-20 Sephadex columns and a Celite liquid-liquid column (50—75% recovery from extraction) before assaying. In 20 normal humans, the average concentration of $1,25\text{-}(OH)_2D_3$ was 64 $\pm$ 12 pgs/ml. In order to measure these low circulating levels, relatively large volumes of plasma are required and the need for multiple column runs hinders the wide application of this assay.

An assay for $1,25\text{-}(OH)_2D_3$ using different techniques is being developed in the Endocrine Unit at the Massachusetts General Hospital utilizing a competitive binding assay for $1,25\text{-}(OH)_2D_3$ with rat plasma vitamin D binding protein. It has already been shown that $1,25\text{-}(OH)_2D_3$ will displace $[^3H]25\text{-}(OH)D_3$ from the vitamin D-OH-D transport protein in rat plasma (123). A standard curve which can detect 10-100 pgs of $1,25\text{-}(OH)_2D_3$ has been obtained, which is sensitive enough to measure $1,25(OH)_2D_3$ in 5 cc of extracted plasma.

Following extraction of vitamin D and its metabolites from plasma, using the method of Bligh and Dyer (124), the extract is dried under nitrogen, and passed over a phenylethoxy-Sephadex column to remove interfering triglycerides and cholesterol. The D and D metabolites are then separated by high pressure liquid chromatography, using the system of Jones and DeLuca which separates $1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_2$ from each other and from other D metabolites (125). Once pure $1,25\text{-}(OH)_2D_3$ is obtained, it is dried under nitrogen, redissolved in ethanol, and measured in the assay system of Belsey et al. (126). The $24,25(OH)_2D_3$ metabolite can also be completely isolated by the liquid chromatography system, and can also be measured separately in the same rat binding protein assay.

Metabolic balance studies are carried out using the techniques of Albright et al. (127), plus chromium sesqui-oxide as a non-absorbable fecal marker. Calcium kinetic studies are carried out using the methods of Neer et al. (83). The various chemical analyses are carried out as described above.
Forearm bone densitometry will be performed in collaboration by Dr. William Harris of the Orthopedic Surgical Research Laboratory using the method of Cameron et al. (128) employing a filtered ^{125}I source and a NaI detector. A specially constructed brace is used to guarantee reproducibility. With this method the coefficient of variation is 3% in our experience. The through-and-through bone biopsies will be performed by hand just below the anterior iliac crest by Dr. D. Patel of the Department of Orthopedic Surgery, using local anaesthesia and an 8-mm internal diameter needle. Undecalcified specimens will be sectioned on a Jung microtome, examined in the Massachusetts General Hospital bone pathology laboratory, and quantitative morphometric analysis of the specimens carried out to provide measurements of trabecular bone volume, osteoid volume, mean cortical thickness, % trabecular surface covered by osteoid, osteoblasts, osteoclasts, double tetracycline label, number of osteoclasts/square millimeter, as well as calculations of the osteoid thickness index and the calcification/apposition index. One month prior to biopsy the patient will receive 250 mg tetracycline BID for 4 doses, and two weeks prior to biopsy the same dose for 8 doses. This pattern of administration causes bone labeling with thin and thick lines which are easily identifiable.

REFERENCES

1. Liddle, G.W.: The Human Adrenal Cortex. ed. N.P. Christy, Harper and Row, New York, p 47 f., 1971.

2. Cope, C.L.: Adrenal Steroids and Disease. Pitman, London, pp. 208-214, 1972.

3. Christy, N.P., op. cit., pp. 359-394.

4. See page 11 of this application.

5. Hansen, J.W. et al.: J Clin Invest 52: 304, 1973.

6. McPherson, G.S.: Acta Orthoped Scand, Suppl 78: 35, 1965.

7. Heaney, R.P. and Skillman, J.: J Clin Endocrinol 33: 661, 1971.

8. Neer, R.M. et al.: J Clin Invest 46: 1364, 1967.

9. Krane, S.M. in Calcium Regulating Hormones. ed. R.V. Talmage, M. Owen and J.A. Parsons, Excerpta Medica, Amsterdam, 1975.

10. Efron, M.L. et al.: New Engl J Med 272: 1299, 1965.

11. Segrest, J.P. and Cunningham, L.W.: J Clin Invest 49: 1497, 1970.

12. Pinnell, S.R., Fox, R. and Krane, S.M.: Biochem Biophys Acta 229: 119, 1971.

13. Askenasi, R.: Biochem Biophys Acta 304: 375, 1973.

14. Askenasi, R.: J Lab Clin Med 83: 673, 1974.

15. Parsons, J.A., Neer, R.M. and Potts, J.T., Jr.: Endocrinology 89: 735, 1972.

16. Parsons, J.A., Reit, B. and Robinson, C.J.: J Physiol 222: 15, 1972.

17. Neuman, W.F.: Calcium, Parathyroid Hormone and Calcitonins. ed. R.V. Talmage and P.L. Munson, Excerpta Medica, Amsterdam, p. 389, 1972.

18. Rasmusen, H., Arnaud, C. and Hawker, C.: Science 144: 1019, 1964.

19. Keiser, H.R. et al.: J Clin Invest 43: 1073, 1964.

20. Barnicot, N.A.: J Anat 82: 233, 1948.

21. Bingham, P.J., Brazell, I.A. and Owen, M.: J Endocrinol 45: 387, 1969.

22. Owen, M. and Bingham, P.: Parathyroid Hormone and Thyrocalcitonin (Calcitonin). ed. R.V. Talmage and L.F. Belanger, Excerpta Medica, Amsterdam, p. 216, 1968.

23. Vaes, G.: Parathyroid Hormone and Thyrocalcitonin (Calcitonin). ed. R.V. Talmage and L.F. Belanger, Excerpta Medica, Amsterdam, p. 318, 1968.

24. Aurbach, G.O. and Chase, L.R.: Federation Proceedings 29: 1179, 1970.

25. Bauer, W., Aub, J.C. and Albright, F.: J Exp Med 49: 145-161 ff., 1929.

26. Selye, H.: Endocrinology 16: 547-558, 1932.

27. Pugsley, L.I. and Selye, H.: J Physiol (London) 73: 113-117, 1933.

28. Jaffe, H.L.: Libman Anniversary Volumes 2: 607-616, 1932.

29. Burrows, R.B.: Am J Anat 62: 237-290, 1938.

30. Shelling, D.H., Asher, D.E. and Jackson, D.A.: John Hopkins Hospital Bulletin 53: 348-389, 1933.

31. Kalu, D.N. et al.: Lancet: 1362, 1970.

32. Genant, H.K. et al.: Am J Med 59: 104, 1975.

33. Wilde, C.D., Jaworski, Z.F., Villanueva, A.R. et al.: Calcified Tissue Research 12: 137-142, 1973.

34. Olah, A.J., Virchow's Arch Pathol Anat 358: 301-308, 1973.

35. Klein, L., Lafferty, F.W., Pearson, O.H. and Curtiss, P.H.: Metabolism 13: 272-284, 1964.

36. Parsons, J.A.: Biochemistry and Physiology of Bone, vol IV. ed. G.H. Bourne, Academic Press, New York, (in press).

37. Sjoberg, H.E.: Hormone Metab Res 1: 32-34, 1970.

38. Mallette, L.E., Sode, J.E., Marx, S.J., Goerges, L.P. and Aurbach, G. D.: J Clin Endocrinol Metab 40: 582-588, 1975.

39. Parsons, J.A. and Reit, B.: Nature 250: 254, 1974.

40. Riggs, B.L. et al.: Clin Endocrinol Metab 2: 317, 1973.

41. Harris, W.H. and Heaney, R.P.: N Engl J Med 280: 193, 1969.

42. Reeve, J., Tregear, G.W. and Parsons, J.A.: Calcified Tissue Research Supplement: Proceedings of the 11th European Calcified Tissue Symposium (in press).

43. Reeve, J., Hesp, R., Williams, D. et al.: Lancet 1: 1035-1038, 1976.

44. Brewer, H.B. et al: Proc Natl Acad Sci USA 69: 3585, 1972.

45. Niall, H.D., Sauer, R.T., Jacobs, J.W. et al.: Proc Natl Acad Sci USA 71:, No. 2: 384-388, 1974.

46. Keutmann, H.R., Niall, H.D., O'Riordan, J.L.H. et al.: Biochemistry 14: 1842-1847, 1975.

47. Keutmann, H.R.: Clin Endocrinol Metab 3: 173, 1974.

48. Parsons, J.A., Rafferty, B., Gray, D. et al.: Calcium-Regulating Hormones 1975: Proceedings of the Fifth Parathyroid Conference. Oxford, United Kingdom, July 21-26, 1974. Excerpta Medica International Congress Series No. 346, Excerpta Medica, Amsterdam, pp. 33-39, 1975.

49. Tregear, G.W., van Rietschoten, J., Greene, E. et al.: Hoppe-Seyler's Z. Physiol Chem Bd 355, X. 415-421, April 1974.

50. Irwin, J.W., Henneman, P.H., Wang, D., and Burrage, W.S.: J Allergy 25: 201, 1954.

51. Sussman, M.L. and Copleman, B.: Radiology 39: 288, 1942.

52. Plotz, C.M., Knowlton, A.I. and Ragan, C.: Am J Med 13: 597, 1952.

53. Hurxthal, L.M. and Sullivan, J.B.: Ann Intern Med 51: 1, 1959.

54. Sprague, R.G., Salassa, R.M., Randall, R.V. et al.: Arch Intern Med 98: 389, 1956.

55. Soffer, L.J., Iannaccone, A. and Gabrilove, J.L.: Am J Med 30: 129, 1961.

56. Boland, E.W. and Headley, N.G.: JAMA 144: 365, 1950.

57. Demartini, F., Grokoest, A.W. and Ragan, C.J.: JAMA 149: 750, 1952.

58. Soffer, L.J. and Bader, R.: JAMA 149: 1002, 1952.

59. Teicher, R. and Nelson, C.: J Invest Dermatol 19: 205, 1952.

60. Reifenstein, E.C.: Southern Medical Journal 49: 933, 1956.

61. Murray, R.O.: Vr J of Radiol 33: 1, 1960.

62. McConkey, B., Frazer, G.M. and Bligh, A.S.: Q J Med 31: 419, 1962.

63. Gallagher, J.C., Aaron, J., Horsman, A. et al.: Clinics in Endocrinol and Metab 355: 1973.

64. Iannaccone, A., Gabrilove, J.L., Brahms, M. and Soffer, L.J.: Ann Intern Med 52: 570, 1960.

65. Skeels, R.F.: J Clin Endocrinol 18: 61, 1958.

66. Jowsey, J.: Am J Med 40: 485, 1966.

67. Storey, E.: Endocrinology 68: 533, 1961.

68. Riggs, B.L., Jowsey, J. and Kelly, P.: Metabolism 15: 773, 1966.

69. Prockop, E.J. and Kivivikko, A.I.: Treatise on Collagen, Part A. ed. B.S. Gould and G.N. Ramachandran, Academic Press, New York, pp. 215-240, 1968.

70. Smith, Q.T. and Allison, D.: Endocrinology 77: 785, 1965.

71. Stoerk, H.C., Peterson, A.C. and Jelinek, V.C.: Proc Soc Exp Biol Med 114: 690, 1963.

72. Williams, G.A., Peterson, W.C., Bowser, E.N. et al.: Endocrinology 95: 707-712, 1974.

73. Kukreja, S.C., Bowser, F., Hargis, G. et al.: Proc Soc Exp Med 152: 358-361, 1976.

74. Fucik, R.F., Kukreja, S.C., Hargis, G.K.: J Clin Endocrinol Metab 40: 152-155, 1975.

75. Eliel, L.P., Chanes, R. and Hawrylko, J.: J Clin Endocrinol 25: 445, 1965.

76. Eliel, L.P., Thompson, C. and Chanes, R.: J Clin Endocrinol 25: 457, 1965.

77. Talmage, R. and Kennedy, J., III: Endocrinology 86: 1075, 1970.

78. Talmage, R., Park, H. and Jee, W.: Endocrinology 86: 1080, 1970.

79. Jee, W., Roberts, W., Park, H., Julian, G. and Kramer, M.: Calcium, Parathyroid Hormone and the Calcitonins. ed. R.V. Talmage, Excerpta Medica, Amsterdam, p. 430, 1970.

80. Hirsch, P.L. and Munson, P.L.: Physiol Rev 49: 548-622, 1969.

81. Talmage, R., Anderson, J. and Cooper, C.: Endocrinology 90: 1185, 1972.

82. Talmage, R. and Anderson, R.: Proc Soc Exp Biol Med 141: 982, 1972.

83. Aldred, J.P., Bobalik, G.R., Smith, C.M. and Bastian, J.W.: Fed Proc 26: 367, 1967.

84. Thompson, J., Palmieri, G., Eliel, L. and Cutler, G.: Endocrinology 83: 470, 1968.

85. Thompson, J. Palmieri, G. and Eliel, L.: Endocrinology 94: 799, 1974.

86. Thompson, J., Palmieri, G., Eliel, L. and Crawford, R.: J of Bone and Joint Surgery 54A: 1490, 1972.

87. Palmieri, G., Dvorak, J. and Bottomley, R.: N Engl J Med 290: 1490, 1974.

88. Verner, J.V., Jr., Engel, L. and McPherson, G.D.: Ann Intern Med 48: 765, 1966.

89. Henneman, P.M., Dempsey, F., Carroll, E. and Albright, F.: J Clin Invest 35: 1229, 1956.

90. Kahn, A., Snapper, I. and Drucker, A.: Arch Intern Med 114: 434, 1964.

91. Harrison, H.G. and Harrison, H.C.: Am J Physiol 199: 265, 1960.

92. Williams, G.A., Bowser, E.N., Henderon, W.J. and Uzgiries, V.: Proc Soc Exp Biol Med 106: 664, 1961.

93. Kimberg, D.V., Baerg, R.D., Gershon, E. and Graudusius, R.: J Clin Invest 50: 1309, 1971.

94. Avioli, L.V., Birge, S.J. and Lee, S.W.: J Clin Endocrinol 28: 1341, 1968.

95. Favus, M.J., Kimberg, D.V., Millar, G.N. and Gershon, E.: J Clin Invest 52: 1328, 1973.

96. Lukert, B.P., Stanbury, S.W. and Mawer, E.B.: Endocrinology 93: 718, 1973.

97. Favus, M.J., Walling, M.W. and Kimberg, D.V.: J Clin Invest 52: 1680, 1973.

98. Carre, M., Ayigbede, O., Miravet, L. and Rasmussen, H.: Proc Natl Acad Sci USA 71: 2996, 1974.

99. Clark, I., Geoffroy, R.F., Bowers, W.: Endocrinology 64: 849-856, 1959.

100. Williams, G.A., Bowser, E.N., Henderson, W.J. and Uzgiries, V.: Proc Soc Exp Biol Med 106: 664-666, 1961.

101. Caniggia, A. and Gennari, C.: Clinical Aspects of Metabolic Bone Disease ed. B. Frame, A. Parfitt and H. Duncan, Excerpta Medica, Amsterdam, p. 333, 1973.

102. Boisseau, V., Hahn, T. and Avioli, L.G.: Clin Res 21: 844, 1973.

103. Pechet, M.M., Bowers, B. and Bartter, F.: J Clin Invest 38: 691, 1959.

104. Laake, H.: Acta Endocrinol (Ibh) 34: 60, 1960.

105. Wajchenberg, B., Quintao, E., Liberman, B. and Cintra, A.: J Clin Endocrinol 25: 1677, 1965.

106. Walser, M.: Am J Physiol 200: 1099, 1961.

107. Massry, S.G. et al.: J Lab Clin Med 71: 212-219, 1968.

108. Suki, W.N., Schwetman, R.S., Rector, R.S., Jr. and Seldin, D.W.: Clin Res 15: 372, 1967.

109. Massry, S., Coburn, J., Chapman, L. and Kleeman, C.: J Lab Clin Med 70: 563, 1967.

110. Lemann, J. et al.: Nephron 7: 117-130, 1970.

111. Goldsmith, R., Siemsen, A., Mason, A. and Forland, M.: J Clin Endocrinol 25: 1649, 1965.

112. Roberts, K. and Pitts, R.: Endocrinology 52: 324, 1953.

113. Relman, A.S. and Schwartz, W.B.: J Clin Invest 31: 656, 1952.

114. Anderson, J. and Foster, J.B.: Clin Sci 18: 437, 1959.

115. Murray, R.M., Peacock, M., Powell, D. et al.: Clin Endocrinol 1: 235-246, 1972.

116. Potts, J.T. and Deftos, J.L.: Duncan's Diseases of Metabolism 7th Edition. ed. P.K. Bondy and L.E. Rosenberg, Saunders, Philadelphia, chapter 20, 1974.

117. Kivirikko, K. et al.: Anal Biochem 19: 249, 1967.

118. Hare, R.S.: Proc Soc Exp Biol Med 74: 148, 1950.

119. Kelleher, P.C. and Bisbee, W.: Program of 5th International Congress of Endocrinology, 1976, Excerpta Medica (in press).

120. Gilman, A.G.: Proc Natl Acad Sci USA 67: 305, 1970.

121. Segre, G.V., Treagear, G.W., and Potts, J.T., Jr.: Development and application of sequence-specific radioimmunoassays for analyses of the metabolism of parathyroid hormone. In, Methods in Enzymology, vol. XXXVII, Hormone Action. Part B: Peptide Hormones. ed. B.W. O'Malley and J.G. Hardman, Academic Press, New York, pp. 38-66, 1975.

122. Brumbaugh, P.F., Haussler, D.H., Bressler, R., Haussler, M.R., Science 183: 1089, 1974.

123. Belsey, R., Clark, M., Bernat, M. et al.: Am J Med 57: 50, 1974.

124. Bligh, E., Dyer, W.: Can J Biochem Physiol 37: 911, 1959.

125. Jones, G. and DeLuca, H.F.: J Lipid Res 16: 448-452, 1975.

126. Belsey, R., DeLuca, H.F., Potts, J.T., Jr.: J Clin Endocrinol Metab 33: 554, 1974.

127. Albright, F., et al.: J Clin Invest 9: 659, 1931.

128. Cameron, J.R. et al.: J Invest Radiol 3: 141, 1968.

116. Potts, J.T. and Deftos, J.L.: Duncan's Diseases of Metabolism 7th Edition. ed. P.K. Bondy and L.E. Rosenberg, Saunders, Philadelphia, chapter 20, 1974.

117. Kivirikko, K. et al.: Anal Biochem 19: 249, 1967.

118. Hare, R.S.: Proc Soc Exp Biol Med 74: 148, 1950.

119. Kelleher, P.C. and Bisbee, W.: Program of 5th International Congress of Endocrinology, 1976, Excerpta Medica (in press).

120. Gilman, A.G.: Proc Natl Acad Sci USA 67: 305, 1970.

121. Segre, G.V., Treagear, G.W., and Potts, J.T., Jr.: Development and application of sequence-specific radioimmunoassays for analyses of the metabolism of parathyroid hormone. In, Methods in Enzymology, vol. XXXVII, Hormone Action. Part B: Peptide Hormones. ed. B.W. O'Malley and J.G. Hardman, Academic Press, New York, pp. 38-66, 1975.

122. Brumbaugh, P.F., Haussler, D.H., Bressler, R., Haussler, M.R., Science 183: 1089, 1974.

123. Belsey, R., Clark, M., Bernat, M. et al.: Am J Med 57: 50, 1974.

124. Bligh, E., Dyer, W.: Can J Biochem Physiol 37: 911, 1959.

125. Jones, G. and DeLuca, H.F.: J Lipid Res 16: 448-452, 1975.

126. Belsey, R., DeLuca, H.F., Potts, J.T., Jr.: J Clin Endocrinol Metab 33: 554, 1974.

127. Albright, F., et al.: J Clin Invest 9: 659, 1931.

128. Cameron, J.R. et al.: J Invest Radiol 3: 141, 1968.