SUMMARY

The hypothesis that certain nonparathyroid tumors associated with hypercalcemia contain a parathyroid hormone-like substance was examined experimentally. Bio-
logic assays of extracts of the tumors in parathyroidectomized rats were negative or
equivocal; factors involved in interpretation of the assays are discussed. Earlier
published estimates of parathyroid hormone-like material in tumors, obtained by im-
munassay of urea extracts of the tumors by a method utilizing direct complement
fixation, were found to be too high because of a nonspecific reaction of the urea extracts.
New evidence supporting the presence of parathyroid hormone-like material in hyper-
calcemic tumors was provided by data showing that HCl extracts of the tumors, like
HCl extracts of parathyroid glands, specifically inhibit the direct complement fixation
by purified bovine parathyroid hormone and anti-bovine parathyroid hormone.
Factors that restrict the inhibition reaction to qualitative identification of para-
thyroid hormone-like substances are discussed.

In recent years attention has been drawn to a syndrome
in patients with malignant neoplasms that is characterized
by marked hypercalcemia without gross or microscopic
evidence of parathyroid enlargement or hyperactivity and
without invasion of osseous tissue. In the majority of
cases, removal of the tumor was associated with the prompt
return of serum calcium to normal, whereas recurrence of
the tumor was followed by reappearance of hypercalcemia
(4). To explain these observations, it has been hypothe-
sized that a blood-borne osteolytic substance is produced
and released by the tumor and that this substance is sim-
ilar or identical in chemical structure to parathyroid hor-
mone. We first became interested in the syndrome and
the hypothesis in 1956, when Dr. John Eager Howard
wrote one of us (P. L. M.) to inquire about the possibility
of testing extracts of hypercalcemic tumors for parathyroid
hormone-like activity in our bioassay method in parathy-
roidectomized rats.

Parathyroid hormone-like activity in the tumor might
escape detection by biologic assay if the concentration of
the activity were too low to meet the minimum dose re-
quirement of the assay. Yet a low concentration of ac-
tivity would not be inconsistent with a high, clinically
significant rate of release of an active substance, owing to
the size of the tumor relative to that of normal or hyper-
active parathyroid glands. The concentration of hormone
in bovine parathyroid glands is a useful point of reference.
The average fresh weight of 1 bovine gland is 120 mg (de-
termined by weighing 900 carefully dissected glands).
The concentration of parathyroid hormone in these glands,
as estimated by bioassay of the classic hot dilute HCl ex-
tract, is approximately 1600 units/gm or 200 units/gland.(4)
The extract, which is customarily prepared with 10 ml of
acid/gm of glands, contains about 160 units/ml.

The biologic assay method (7), in which rats on a low-
calcium diet are parathyroidectomized and immediately
thereafter injected s.c. with a standard or unknown prep-
paration, requires a minimum dose of 10–15 units/rat to
produce a statistically significant elevation above the con-
trol level of serum calcium in a group of 5 test rats. There-
fore, injection of 0.1 ml of bovine parathyroid extract
normally would yield a detectable effect. (Limited tests
of extracts of normal human parathyroid glands and ade-
nomas indicate that they do not contain any higher con-
centration and may contain a lower concentration of hor-
mone than bovine glands.) In order for a similar extract
of tumor to produce a detectable effect in our test rats at

1 Contribution No. 372 of the Graduate Department of Bio-
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(A-1787), USPHS, and the American Cancer Society (E-222).
2 Special Research Fellow, National Institute of Arthritis and
Metabolic Diseases, USPHS.
3 American Cancer Society Professor of Biochemistry, Brandeis
University, Waltham, Mass.
4 Unpublished observations by P. L. Munson and B. A. San-
born. The lower concentration reported by Kenny et al. (6) was
for undissected glands as received from the supplier. When proper
allowance is made for nonparathyroid tissue in undissected glands,
the 2 reports are in reasonable agreement.

Evidence for Parathyroid Hormone in Nonparathyroid Tumors
Associated with Hypercalcemia1

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Massachusetts; and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts)
the usual injection volume of 1.0 ml, the concentration of hormone in the tumor would need to be one-tenth as high as it is in bovine glands, or if 4.0 ml, an excessive volume, were injected, the concentration of hormone would need to be 0.025 as high as in parathyroid glands.

There are several other considerations to be taken into account in the interpretation of results of a biologic assay for parathyroid hormone (or any other active agent). One that is particularly relevant when testing a crude extract such as that of a tumor, in which the concentration of parathyroid hormone-like activity may be low, is the limited significance of a barely detectable response above the control level. A classic criterion for determining qualitative nonidentity of 2 similar activities in biologic assay is the appearance of nonparallel dose-response curves. In the absence of such a test, which requires administration of the suspect unknown at 2 or more distinguishable dose levels in parallel with several dose levels of the reference standard, an important clue to the nature of the unknown is unavailable. In order to be able to inject the higher of the 2 dose levels required for the parallelism test, a rather high concentration of parathyroid hormone-like activity must be present in the extract, or, alternatively, the activity must be concentrated by some means.

By a mechanism not yet understood, the injection of cysteine (0.12 M) with parathyroid hormone in the bioassay enhances the activity of the hormone about 3-fold, which reduces the dose requirement by a like factor. However, the phenomenon is not a clear gain in the detection of minimum amounts of hormone, since injection of cysteine alone, in comparison with uninjected controls or controls injected with acidified physiologic salt solution, has a small serum calcium-raising effect which, although not usually statistically significant in individual experiments, is significant when a series of experiments are analyzed together (7).

Finally, it must be emphasized that even if dose-response curves for parathyroid hormone and tumor extracts pass the test for parallelism it does not prove that the tumor extract contains parathyroid hormone, only that it contains an agent with similar activity. On the other hand, parallelism in a bioassay provides much stronger evidence for similarity, if not identity, than a minimum effect, which is all that could be hoped for from tumors containing a low concentration of the active agent.

The purpose of the foregoing analysis of the limitations of biologic assay is to place in perspective the results we have obtained from the bioassay of tumor extracts. Our earlier assays of the hypercalcemic tumors supplied by Dr. Howard and others were negative or equivocal (A. D. Kenny and P. L. Munson, unpublished observations). The positive test that we published earlier this year (9), which was based on results statistically significant only at the level of \( P < 0.05 \), was not regarded as strong evidence but only "consistent with the presence of parathyroid hormone" in the tumors.

In order to make an adequate test for parathyroid hormone in the tumors in question it was necessary to turn to a method sensitive to much smaller amounts than are required for the bioassay in rats. An immunochemical method based on complement (C') fixation between parathyroid hormone and specific rabbit anti-bovine parathyroid hormone appeared to meet this requirement (8).

The immunologic difference between human and bovine parathyroid hormone, shown by the failure of hormone extracted from human parathyroid glands by phenol or by dilute HCl to fix C' directly with the antibody, was a problem. It appeared to be solved by the discovery that extracts of human parathyroid glands made with aqueous 8 M urea did fix C' directly with anti-bovine parathyroid hormone at an antibody concentration approximately 7 times greater than was necessary for the homologous reaction. Furthermore, it was found that the phenol and HCl extracts of human glands contained forms of the hormone that were antigenically similar, though not identical to the bovine hormone, since the homologous C'-fixation reaction was inhibited by these extracts (9).

The nonidentity of human and bovine parathyroid hormones has also been demonstrated immunologically by Berson et al. (3).

Nonparathyroid tumors from 6 patients with hypercalcemia were extracted (also metastases from 3 of the patients) with urea. All 9 of the extracts fixed C' directly with the anti-bovine parathyroid hormone. Some of the tumors were also extracted with phenol and HCl and the extracts of all tumors thus examined inhibited the homologous C'-fixation reaction (9).

Evidence for the specificity of the direct C'-fixation reaction was provided by negative tests with urea extracts of normal tissues from the 6 tumor patients and of tumors from other patients without hypercalcemia. In addition, antibody-absorption experiments indicated removal of anti-parathyroid hormone antibody from solution by the tumor extract. Evidence for the specificity of the C'-fixation inhibition reaction was provided by the lack of inhibition of 3 nonparathyroid immune systems by extracts that inhibited the parathyroid system (9).

Quantitative estimates of the amount of parathyroid hormone in 6 tumors and 3 metastases, based on the

### TABLE 1
**BIOASSAYS OF TUMOR EXTRACT**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>EXPERIMENT I</th>
<th>EXPERIMENT II</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Plasma calcium(^a) (mg/100 ml)</td>
<td>Urine P(^c) (mg/rat/hr)</td>
</tr>
<tr>
<td>HCl control</td>
<td>6.1</td>
<td>0.14</td>
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<td>Cysteine control</td>
<td>7.1</td>
<td>0.11</td>
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<td>Parathyroid extract, 15 USP units</td>
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<tr>
<td>Parathyroid extract, 60 USP units</td>
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<tr>
<td>Tumor No. 2 extract(^b)</td>
<td>7.8</td>
<td>0.15</td>
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</table>

\(^a\) Mean value, 5 parathyroidectomized rats per group, with 1 exception. Standard errors for calcium: Experiment I, 0.66; Experiment II, 0.47–0.52; for inorganic phosphorus (P\(_i\)), 0.18.

\(^b\) Cysteine (0.12 M) was added to the extract just before administration to the assay rats. The volume of cysteine solution or tumor extract injected per rat was 2.5 ml in Experiment I and 4.0 ml in Experiment II.
immunoassay of urea extracts, were reported to range between 0.9 and 39 μg/gm, or in units, 3-135/gm. Extracts of 3 of the tumors estimated to contain 35-45 units/gm by immunoassay were bioassayed in parathyroidectomized rats. When the results of the 3 assays were combined, the increase in serum calcium was barely significant statistically at \( P < 0.05 \). If valid, the results would have indicated an amount of hormone in the tumor

### TABLE 2

% Inhibition of C' Fixation by Dilute Acid Exacts

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Dilution of inhibitor</th>
<th>CMC-PTH anti-PTH</th>
<th>Seph-PTH anti-PTH</th>
<th>Egg albumin-anti-egg albumin</th>
<th>Human growth hormone-anti-growth hormone</th>
<th>Dogfish pepsinogen anti-pepsinogen</th>
<th>Rickettsiophage DNA anti-DNA</th>
<th>Horse spleen ferritin anti-ferritin</th>
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<tr>
<td>Tumors not associated with hypercalcemia</td>
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* CMC-PTH, parathyroid hormone purified on carboxymethylcellulose following Sephadex G-100 (donated by Drs. J. T. Potts, Jr., and G. D. Aurbach). The C' fixation being inhibited was that obtained with 0.65-1.25 μg of hormone and a dilution of antisera of 1:500-1:3000. No important qualitative or quantitative differences were noted in the C' fixation curves obtained with this antigen and the highly purified hormone (Seph-PTH) used in the original study (9).

* g * Seph-PTH, parathyroid hormone purified on Sephadex G-50 (as described in Ref. 10). The C' fixation being inhibited was that obtained with 0.6 μg of hormone and a dilution of antisera of 1:500-1:3000.

* Three different HCl extracts of the same tumor were used. The 1st dilution of extract was equivalent to 150-300 mg of tumor.

* Four different HCl extracts of the same tumor were used in this series of experiments. The 1st dilution of extract was equivalent to 100-300 mg of tumor.

* Extracts prepared exactly the same as for tumors. The 1st dilutions of the liver and kidney extracts were equivalent to >300 mg of tissue, while the 1st dilution of the parathyroid gland extract was equivalent to only 1 mg of tissue.

* Two different tumors were extracted, a pancreatic adenocarcinoma and an adenocarcinoma of the rectosigmoid. The 1st dilutions of these extracts were equivalent to approximately 300 mg of tissue.
extract by bioassay consistent with the results by immunoassay. However, we did not regard the bioassay as a thoroughgoing confirmation of the immunoassay results, for the reasons outlined earlier.

There are 3 types of experiments that have led us to revise our conclusions (9) concerning the concentration of parathyroid hormone-like activity in the tumors associated with hypercalcemia.

First, additional biologic assays failed to confirm the high estimates by immunoassay. In these experiments, higher dose levels of the extract of a tumor were used than in the experiment published, so that clearly positive results should have been obtained if the results by immunoassay had been correct. The higher doses were achieved by concentrating the urea extracts by a method similar to that used by Aurbach for preliminary purification of phenol extracts of bovine parathyroid glands (1).

To test the applicability of this procedure to urea extracts, ground, frozen bovine parathyroid glands (undissected) were extracted with aqueous 8% urea (20 ml/gm) and the urea extract was concentrated by precipitation with impurities with acid-acetone-NaCl, followed by precipitation of the activity with ether. The estimated potency in the bioassay of a sample of the ether precipitate taken up in 0.01 N HCl led to a value of 110 ± 1.25 μg (385 units) of parathyroid hormone per gm of original tissue, which agrees well with our previous estimates of hormone content of undissected bovine glands. The value obtained by immunoassay of the ether precipitate by direct C' fixation was 60 μg/gm, in fair agreement with the bioassay.

The results of the 2 biologic assays of the partially purified urea extract of tumor are given in Table 1. There was no significant effect (above the cysteine control) on either plasma calcium or urine phosphate in Experiment I, and in Experiment II, although the effect on serum calcium approached statistical significance, P was > 0.05. The dose of extract given was such that there should have been a clearly positive effect if the tumor had contained as much as 2–4 μg of parathyroid hormone per gm of tissue, a level lower than that estimated from immunoassays by direct complement fixation of urea extracts (10 μg/gm) (9). Although it has been shown that the biologic activity of parathyroid hormone in vivo may be low or negligible with preservation of full immunologic activity (10), there was no reason to believe that this was the cause for the discrepancies between the 2 different assay methods applied to tumor extracts.

Secondly, a preliminary experiment by R. Melick, J. T. Potts, Jr., and G. D. Aurbach (personal communication), using the radioimmunoassay of Benson et al. (3) to test urea extracts of hypercalcemic tumors, including 2 tumors previously assayed by us, indicated that there was no more than 0.025 as much parathyroid hormone-like activity present as we had originally estimated by direct C' fixation.

Thirdly, it was found that when urea extracts of tumors from 3 of the original hypercalcemic patients (Nos. 1, 2, and 4) were incubated with C' and numerous rabbit sera, immune and nonimmune, at dilutions of 1/100–1/200, a reaction simulating C' fixation took place with some of the sera, although not with all of them. The presence or extent of the reaction was not correlated with the antibody content of the serum. When tumor extracts were incubated with specific anti-parathyroid hormone in the same experiment in which control antisera were used, the amount of C' fixed with the anti-parathyroid hormone was usually but not invariably greater than that obtained with the unrelated sera. It thus became apparent that a reaction was occurring between some component of the C' system, rabbit serum, and urea extracts of tumors from hypercalcemic patients that was not specific for anti-parathyroid hormone. Since the antigen controls were consistently not anti-complementary, the reaction required rabbit serum. We have not determined the nature of this complicating reaction. However, the reaction between anti-parathyroid hormone and the urea extracts of tumors does not merely reflect the high protein content of the antigen, as no such reaction has been seen between anti-parathyroid hormone and many different human sera at low dilution where the nonspecific protein content exceeds that of the tumor extracts.

These 3 lines of evidence required us to revise our previously published estimates of parathyroid hormone-like material in tumors. The question could also be raised as to the existence of any such material in the tumors.

Data from biologic assays, for reasons already given, are inadequate either to support or to deny the presence of parathyroid hormone in the hypercalcemic tumors.

The radioimmunoassay data of Melick, Potts, and Aurbach, while provocative, also do not support the presence of parathyroid hormone in the tumors in any unequivocal manner. These unpublished preliminary data, kindly communicated to us by the investigators, showed a positive reaction with urea extracts of tumors at the borderline of sensitivity of the radioimmunoassay method. However, the reaction was unlike that of parathyroid hormone in that the use of increased amounts of the urea extracts did not cause a consistent or parallel increase in the extent of the reaction.

Our conclusion that a substance immunologically similar to or identical with human parathyroid hormone is present in hypercalcemic tumors now rests solely on new experiments demonstrating the inhibitory effect of HCl extracts on C' fixation by bovine parathyroid hormone and anti-bovine parathyroid hormone.

Dilute acid extracts were chosen for study because of the previous observation that treatment of bovine parathyroid hormone with acid converts the molecule from an antigen that fixes C' directly with antibody to one that does not fix C' directly but still reacts with antibody as evidenced by inhibition of C' fixation (9). For these experiments, a sample of the purest parathyroid hormone currently available, purified further following gel filtration through Sephadex G-100 (2) by chromatography on carboxymethylcellulose, was used as the antigen against which the tumor extracts were to be tested by competition for antibody. This preparation was kindly donated by Drs. J. T. Potts, Jr., and G. D. Aurbach. Similar results were obtained with 2 anti-parathyroid sera produced in different rabbits against parathyroid hormone purified by gel filtration (10). Dilute HCl extracts of 3 of the original tumors (Nos. 1, 2, and 4), which had been stored
The relationship of antigen concentration to maximum C' fixation

<table>
<thead>
<tr>
<th>Immune system</th>
<th>Approximate molecular weight of antigen (pg/6ml)</th>
<th>Amount of antigen added to give maximum C' fixation (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathyroid hormone</td>
<td>8,500</td>
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</tr>
<tr>
<td>Bovine pancreatic ribonuclease</td>
<td>13,500</td>
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</tr>
<tr>
<td>β-Chain human hemoglobin</td>
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<td>Human hemoglobin</td>
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<td>Dogfish pepsinogen</td>
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</table>

* These values are given for a dilution of antibody which will give 70-80% maximum C' fixation.

b Molecular weight unknown.

at −20°C, were prepared as previously described (9). It was reasoned that if the reaction between pure bovine parathyroid hormone and its homologous antibody could be inhibited by HCl extracts of tumors obtained from the hypercalcemic patients, but not by similar extracts of normal tissues or tumors from nonhypercalcemic patients, and if the C'-fixation inhibition reaction could be shown to be specific for the parathyroid immune system, then it would be reasonable to conclude that the tumors contained a substance immunochemically similar to parathyroid hormone.

The results, shown in Table 2, indicate consistent inhibition of the parathyroid hormone immune system by acid extracts of 3 tumors associated with hypercalcemia. Similar extracts of normal tissues and of tumors not associated with hypercalcemia did not inhibit this system. Each inhibition mixture was controlled at all dilutions and any tubes showing any interference with C' activity were excluded. The reason for the variability in amount of inhibition observed from one experiment to another is not clear. However, it may be due in part to differences in the concentration of the inhibitor in different extracts prepared from the same tumor on different dates or to changes on storage of the extracts. The lack of any significant inhibition of the 5 nonparathyroid immune systems tested is evidence for the specificity of the inhibition of the parathyroid hormone immune system. The occasional value of 10% or less inhibition of nonparathyroid systems shown in Table 2 is not significant. However, the unlikely possibility is not excluded that extracts of the tumors combine with parathyroid hormone and prevent its interaction with antibody, a result that would also inhibit C' fixation.

The data in Table 2 on the inhibitory effect of acid extracts of tumors, although indicative of the presence of a parathyroid hormone-like substance in the tumors, are not adequate for quantitative estimates. It was observed that the maximum C' fixation by pure parathyroid hormone reacting with several different rabbit anti-parathyroid hormone sera required 1.5–2.0 µg of antigen. As shown in Table 3, this exceeds, by a factor ranging between 6 and 180, the amount of other antigens usually required for maximum C' fixation by this technic. The outstanding exception, other than parathyroid hormone, with a requirement of 5.5 µg of antigen for maximum fixation, was the denatured β-chain of human hemoglobin (5), which aggregates in aqueous media. We have previously published data indicating that aggregation of parathyroid hormone occurs in neutral buffers, and suggest that the large amount of hormone required to reach maximum C' fixation reflects, at least in part, this property of the antigen in solution. Since it was known that the direct C'-fixing antigen was probably aggregated, but since the physical state of the acid-extracted inhibitor or the stoichiometry of the inhibition were not known, it was not considered justifiable to estimate the hormone content of the tumors by the inhibition reaction of acid extracts using calculations based on stoichiometry. Additional experiments with purified human parathyroid hormone as a standard, when it becomes available, and further studies of acid extracts of human parathyroid glands may make semi-quantitative estimates feasible in the future.

The limitations of the inhibition reaction, using dilute HCl extracts, and of direct C' fixation, using urea extracts, have been emphasized in this paper. However, it is important to point out that direct C' fixation with parathyroid hormone, partially or highly purified from phenol extracts of bovine glands, and anti-parathyroid hormone is a specific reaction (8) that can be used to obtain valid estimates of immunologically active hormone. Parathyroid hormone was found not to react with antisera directed against other antigens and anti-parathyroid hormone was found not to react with other linear polypeptides (A. H. Tashjian, Jr., and L. Levine, unpublished observations).

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