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Some physical characteristics of an adenylate cyclase-stimulating factor(s) associated with humoral hypercalcemia of malignancy

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SOME PHYSICAL CHARACTERISTICS OF AN ADENYLATE CYCLASE-STIMULATING FACTOR(S) ASSOCIATED WITH HUMORAL HYPERCALCEMIA OF MALIGNANCY

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ABSTRACT

SOME PHYSICAL CHARACTERISTICS OF AN ADENYLATE CYCLASE-STIMULATING FACTOR(S) ASSOCIATED WITH HUMORAL HYPERCALCEMIA OF MALIGNANCY

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Hypercalcemia is the most common paraneoplastic syndrome. There are two pathophysiologic groups of patients manifesting malignancy associated hypercalcemia. One group suffers from tumors that metastasize to the skeleton and cause Local Osteolytic Hypercalcemia. The other group suffers from tumors that lack skeletal involvement and cause Humoral Hypercalcemia of Malignancy. A PTH-like, adenylate cyclase-stimulating factor(s) has been implicated as the causative agent in Humoral Hypercalcemia of Malignancy. As part of the effort to isolate and purify this factor, studies were undertaken to define some of its physical characteristics. It was found that, like bPTH(1-34), this adenylate cyclase-stimulating factor(s) is not sensitive to severe reducing conditions. Also similar to PTH, the factor(s) was found to be sensitive to oxidation by hydrogen peroxide. However, it is oxidized much more gradually than is bPTH(1-34). It was found that the factor(s) does not irreversibly lose its bioactivity when exposed to pH's ranging from 3 to 10. Finally, initial attempts to generate an active peptide fragment by partial digestion with cyanogen bromide and initial attempts to ascertain whether the factor(s) is a glycoprotein, using lectin affinity columns, are described.

DEDICATED TO MY PARENTS

Thank you for all the love and the guidance
that you have given to me all these years.

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INTRODUCTION

In 1921 Kramer and Tisdall introduced a chemical method for the direct measurement of serum calcium (1). With the modifications of Clark and Collip (2) this test became routinely available for use in both clinical medicine and research. Not very long after the advent of this technology, Zondek et. al. (3) recorded the first observations of hypercalcemia in a patient with malignancy. Since this report, much attention has been given to malignancy - associated hypercalcemia (MAHC). Today it is recognized to be the most common paraneoplastic complication.

Ten to twenty percent of all cancer patients (4) and fifteen to forty percent of patients with selected neoplasms (5) develop hypercalcemia at some point in their disease. This makes MAHC the second most common cause of hypercalcemia in the general population; primary hyperparathyroidism is the most common cause. Since the latter is often an asymptomatic, ambulatory disease (6,7), and since cancer patients are more frequently hospitalized, MAHC becomes the most common cause of hypercalcemia among inpatients (8,9). Mundy, Cove, and Fisken have looked at the overall incidence of MAHC most closely (6). They studied 207 patients presenting with hypercalcemia in Birmingham, England, a community of approximately one million people. MAHC accounted for thirty-five percent of all hypercalcemia patients in this population, an incidence rate of 150 new patients per million population per year (6,9).

Although hypercalcemia is a frequent companion of malignancy, it is not a ubiquitous one. That is to say, it seems to be associated with only certain types of cancer. For example, hypercalcemia rarely accompanies gastrointestinal cancers (9), and although associated with squamous lung cancer, it is rarely associated with either adenocarcinoma or small cell carcinoma of the lung (10). The syndrome has been repeatedly shown to occur almost exclusively with a specific subset of malignancies (8 - 15). These are: breast carcinoma, multiple myeloma, squamous tumors of lung, esophagus, head and neck, skin, cervix, and vulva, renal cell carcinoma, urothelial carcinomas, malignant lymphomas including Burkit's lymphoma, ovarian carcinoma, and lymphosarcomas. This set of neoplasms can be classified further based on the presence or absence of skeletal metastases. Mundy and Martin (9) use these two critera, histology and metastatic involvement, to define 3 groups of tumors associated with hypercalcemia: solid tumors without skeletal metastases, solid tumors with skeletal metastases, and hematologic malignancies. Stewart et. al. (8) combine the hematologic malignancies with the solid tumors and simply consider two groups: those tumors associated with skeletal metastases and those tumors with limited or no skeletal metastases. Looking at the tumors either way, it is clear (10-15) that breast cancer, multiple myeloma and lymphomas are the tumors that cause hypercalcemia in the presence of skeletal metastases. The squamous, renal, urothelial and ovarian tumors are those that cause hypercalcemia in the absence of skeletal involvement.

These distinctions have implications for pathogenesis. Neoplasms that require an extensive skeletal tumor burden to cause hypercalcemia most likely exert their hypercalcemic effects locally, within and around metastatic lesions in bone. On the other hand, the fact that some neoplasms can cause

hypercalcemia without a skeletal tumor burden suggests that they may exert their hypercalcemic effects over a distance, perhaps via a humoral mechanism. Both of these general mechanisms will be discussed below. However, it is necessary first to consider their common endpoint, increased bone resorption.

In both types of MAHC, with or without bony metastases, excess calcium comes from the skeleton (8,9). Normal calcium homeostasis relies on the balance struck between the gut absorption, the skeletal turnover, and the renal excretion of the ion (5,6,16). Several lines of evidence have established that increased skeletal bone resorption and neither gut hyperabsorption nor decreased renal clearance of calcium accounts for the hypercalcemia associated with neoplastic disease. First, in a study of fifty unselected patients with MAHC, Stewart et al. found mean plasma 1,25 dihydroxy-vitamin D (1,25 $(OH)_2$ Vit. D) values to be markedly depressed in both patients with bony metastases and in patients without bony metastases (14). Since 1,25 $(OH)_2$ Vit. D is the major factor mediating intestinal absorption of calcium (5,17) and since calcium is malabsorbed in its absence (5,18), it is, therefore, improbable that calcium hyperabsorption is the cause of MAHC. Further evidence comes from the observations that dietary calcium restriction does not mitigate the hypercalcemia in either humans with MAHC (19,20) or in animal models of MAHC (21.22). Finally, Coombes et. al. (15), using radioactive calcium intestinal absorption techniques directly showed that both groups of patients with MAHC had depressed levels of intestinal calcium absorption. Hence, the excess calcium in MAHC does not come from the diet.

The evidence against decreased renal excretion of calcium is equally compelling. In fact, Stewart et. al. (14) found just the opposite; fasting urinary calcium excretion was increased in patients with MAHC. It was higher in these

patients than in either normocalcemic cancer patients or patients with primary hyperparathyroidism. Similar observations have been noted repeatedly (10,13,15,23,24). For a given filtered load, the nephron, rather than holding on to calcium, excretes greater amounts. This is true whether one measures fractional calcium excretion or twenty-four hour urine calcium excretion.

The lack of calcium hyperabsorption by the intestine or excessive reabsorption by the nephron is indirect evidence that the hypercalcemia associated with cancer is a result of stepped-up bone resorption. There is also direct evidence. Several studies of bone histology and quantitative bone histomorphometry have demonstrated enhanced bone destruction and increased bone resorption in MAHC with or without local metastases (25-28). Studies of calcium kinetics in hypercalcemic cancer patients (15,19) have also indicated an increase in bone resorption. In fact, one careful study of a patient with multiple myeloma (19) was able to document a parallel relationship between increased serum calcium concentrations and increased bone resorption. Finally, calcitonin (29) and mithramycin (30) are two drugs which have been shown to be efficacious in treating MAHC. Since each of these agents inhibits bone resorption and since each does so through a separate and distinct mechanism, their dual efficacy is strong supporting evidence that increased bone resorption is the cornerstone of MAHC.

LOCAL OSTEOLYTIC HYPERCALCEMIA

One of the two major pathophysiologic groups of cancers associated with hypercalcemia consists of those tumors with extensive skeletal metastases. These tumors seem to cause hypercalcemia through the local osteolytic effects of the metastatic lesions (8,9). Therefore, this subset of MAHC is referred to as

Local Osteolytic Hypercalcemia or LOH (8). Neoplasms included in this group are a common source of MAHC, accounting for anywhere from twenty to ninety percent of all cases, depending on the study (13,14). Histologically they include solid tumors, which are almost exclusively breast carcinomas, and hematologic cancers, most often multiple myeloma and less commonly lymphomas and lymphosarcomas (8,9,14). Despite their common occurrence, the mechanisms responsible for hypercalcemia in these neoplasms are less well defined than the mechanisms at play in the second major group, tumors without bone metastases. Gutman et. al.(11) published the first large series of patients with MAHC. Most of these patients had either breast cancer or multiple myeloma and they all had extensive skeletal tumor burdens. They were patients displaying LOH. Because of this universal skeletal involvement, the authors concluded that the hypercalcemia was caused by direct destruction of bone by the enlarging metastatic lesions. Since this report, it has been recognized that in both hematologic tumors (31) and in solid tumors(32) there is some correlation between the extent of skeletal tumor involvement and the presence of hypercalcemia. For example, it has been observed that patients with breast cancer and certain types of lung cancer usually develop hyper calcemia only late in their disease, after they have extensive osteolytic bone destruction (32). However, it has also become increasingly clear that any correlation between skeletal metastases and hypercalcemia is, at best, a rough one. First, not all tumors that metastasize to bone cause hypercalcemia. For instance, small cell carcinoma is the lung cancer most frequently associated with bone metastases, yet it rarely causes hyper calcemia (10). Second, in a study of thirty-three myeloma patients (31), Durie et. al. could not demonstrate a direct positive correlation between the degree of bone involvement and the serum calcium

concentration. Finally, Ralston et. al., in a histologically diverse series of patients with apparent LOH, actually found an inverse correlation between the number of bone metastases and the serum calcium level (33). All of the above data suggest that the original notion that metastatic lesions produce hypercalcemia purely by destroying bone as they expand is overly simplistic. Many investigators now believe that the ability of a metastatic tumor to cause LOH is initimately related to its production of local or paracrine factors that have the ability to alter normal bone physiology and stimulate bone resorption (8).

The most common mechanisms involved in LOH are those associated with solid tumors , and the majority of solid tumors causing LOH are carcinomas of the breast (9,13). Breast cancer is a very common form of malignancy in the general population and anywhere from sixty to ninety percent of patients with advanced disease have skeletal metastases (32,34-36). Thirty percent of all breast cancer patients develop hypercalcemia (32). As in most MAHC, hypercalcemia in breast cancer is caused by increased bone resorption (9,15). There is evidence that this may occur on the basis of increased osteoclast numbers and activity (25), or that it may occur through the direct resorption of bone tissue by malignant cells (28). Galasko and Bennett (26,37) have suggested that, in fact, both these mechanisms may play a part. However, they feel that osteoclastic bone resorption is quantitatively the more important process.

It seems likely that osteoclast activation in LOH is mediated by locally active, diffusible factors (8). Galasko and Bennett (37) initially suggested that this factor(s) might be PGE_2. It is known that PGE_2 is a potent stimulator of in vitro bone resorption (38), and it has been shown that tumor cells can secrete PGE_2 (39). Furthermore, co-culture experiments have demonstrated that

prostaglandin release by tumor cells in culture has the ability to cause resorption in adjacent bone organ cultures (40-42). Another possibility put forth by Mundy and Martin (9) is that these local diffusible factors are produced not by the tumor, but by lymphocytes and/or monocytes attracted to the metastatic site by a cellular immune response induced by the tumor. As will be discussed below, activated lymphocytes release lymphokines with potent in vitro bone-resorbing activity. Monocytes have been shown both to release prostaglandins (43) and also to resorb bone directly (44,45).

All of the mechanisms mentioned above may play some role in the development of hypercalcemia associated with solid tumor skeletal metastases. At this time, it is not known which one(s) plays the most important role. A more complete understanding awaits further elucidation of both normal bone physiology and those alterations occurring in and around metastatic lesions.

The other group of malignancies associated with LOH is the hematologic cancers. In Myers' series on MAHC (13), this group comprised 20% of all hypercalcemic cancer patients. Multiple myeloma is the most commonly occurring tumor in this group. Most patients with myeloma suffer from extensive bone destruction, either in the form of discrete punched-out lesions or diffuse osteopenia (9). Twenty to thirty percent of those patients become hypercalcemic (46). The mechanism of LOH in multiple myeloma is better understood than that associated with solid tumors. It seems to be associated with the production of a family of osteoclast activating factors (OAFs) by the malignant cells. OAFs are a group of lymphokines that have been shown to cause bone resorption in *in vitro* bioassays (49).

Histological studies have demonstrated that hypercalcemia in myeloma is associated with markedly increased osteoclast numbers and activity (47,48).

Since it was known that activated lymphocytes produce bone-resorbing factors (49) and since myeloma cells are descended from B lymphocytes, Mundy and his associated reasoned that myeloma cells might also produce these bone-resorbing factors. They subsequently showed that tissue culture medium conditioned by myeloma cell lines, by aspirated bone marrow cells and by bone explants of iliac crest, all taken from patients with multiple myeloma,contained bone-resorbing factors with chemical and biological characteristics similar to those of OAFs (47,50,51). These findings are not restricted to multiple myeloma. OAF-like activity has been demonstrated in patients with Burkitt's lymphoma (50), lymphosarcoma (52), and a retrovirus associated T-cell lymphoma (53,54). Therefore, although there are reports (55,56) of lymphomas that secrete $1,25(OH)_2$ Vit. D systemically, it seems likely that most hematologic malignancies produce hypercalcemia via the local elaboration of OAFs by malignant cells present in the skeleton. Recently, two of these OAFs have been identified. Interleukin 1 has been purified from myeloma cell culture medium , and identified as the factor responsible for *in vitro* bone resorbing activity seen with such medium (57). Also tumor necrosis factor, another lymphokine elaborated by myeloma cells , has been shown to posses *in vitro* bone-resorbing activity(58).

Biochemically, patients harboring both solid and hematologic tumors causing LOH generally have reduced levels of circulating immunoreactive parathyroid hormone (iPTH), reduced concentrations of plasma $1,25\ (OH)_2$Vit. D, normal levels of serum phosphate, and normal or reduced levels of nephrogenous cyclic AMP (14). This pattern suggests parathyroid gland suppression, which is expected in the face of elevated serum calcium levels. However, it stands in

contrast to the findings encountered in the second group of patients, those with no skeletal metastases.

HUMORAL HYPERCALCEMIA OF MALIGNANCY

The second major pathophysiologic group consists of the squamous, renal, urothelial and ovarian tumors which have the ability to cause hypercalcemia without metastasizing to the skeleton. These tumors seem to act through a humoral mechanism. Hence, this syndrome has become known as the Humoral Hypercalcemia of Malignancy or HHM (14). HHM is probably less common than LOH, but its exact incidence is unclear. Stewart et. al. (14), in a study of fifty consecutive patients with MAHC, found that eighty percent of them had HHM. However, this study drew heavily from a VA hospital population and had a correspondingly low incidence of breast cancer (6%). Bender and Hansen (10) studied two hundred consecutive cases of bronchogenic carcinoma and found the overall incidence of HHM to be seven percent. This represented fifty-six percent of all patients who developed hypercalcemia in that selected population. The largest published series on MAHC (430 cases collected over 5 years) found that HHM accounted for thirteen percent of all patients with MAHC (13). There are no direct observations on the incidence of HHM in the unselected general population.

Historically, the first reported case of HHM was that of a fifty-seven year old man with bronchogenic carcinoma, hypercalcemia, and hypophosphatemia. This patient was included in Gutman et. al.'s 1936 paper on serum calcium values in hyperparathyroidism, Paget's Disease and MAHC (11).

This patient had a localized area of Paget's disease in his right femur, but no skeletal tumor involvement. The authors concluded:

> It is believed the hypercalcemia in this case was not due to the localized and relatively inactive Paget's disease but to complicating factors as yet wholly obscure.

The first clue as to the nature of these "obscure complicating factors" came from Albright in 1941 (59). He described a patient with a renal carcinoma and a solitary metastasis in the right ilium. The patient was hypercalcemic and hypophosphatemic, both of which corrected temporarily when the patient's tumor was treated with radiation. Combining the observations: 1. that the man was hypophosphatemic rather than hyperphosphatemic (the expected result of any skeletal destruction by the tumor) and 2. that the hypercalcemia was corrected when the tumor was treated, Albright suggested that the hypercalcemia resulted from a tumor-derived humoral factor. Specifically, he thought the tumor might be producing parathyroid hormone (PTH).

Following Albright's paper, there were occasional similar case reports (60). However, the next milestone in the characterization of HHM as a distinct entity came in 1956. Conner, Thomas, and Howard (61) reported two patients with hypercalcemia and hypophosphatemia associated with lung cancer. In both patients, surgical resection of the tumor brought about the normalization of serum calcium and phosphate levels. In one of the patients, tumor recurrence four months later was accompanied by the recurrence of the hypercalcemia and hypophosphatemia. Both patients were shown to have histological evidence of excessive bone resorption in sites uninvolved by tumor. Later that same year, Plimpton and Gellhorn reported similar results (20). They described ten patients

who also had hypercalcemia and hypophosphatemia in association with various malignancies. None of the ten had skeletal tumor involvement as assessed by roentgenographic survey and/or autopsy observations, and all had normal parathyroid glands as assessed by neck exploration and/or autopsy examination. Three of the ten patients underwent resection of their cancer which resulted in prolonged normalization of serum calcium and phosphorus values. One of these three suffered a recurrence and subsequently experienced a return of her hypercalcemia. Taken together, these two papers documented a direct link between the presence of hypercalcemia and the extra-skeletal primary tumor in the patients studied. Both sets of authors concluded that there exists a subset of patients with MAHC in whom excessive bone resorption is caused by a humoral mechanism, and not by local osteolytic events.

The humoral syndrome, which became known as "ectopic hyperparathyroidism" or "pseudohyperparathyroidism" (62,63) was considered a rare entity until Lafferty published the first large series of patients in 1966 (12). The author defined pseudohyperparathyroidism as the the occurence of hypercalcemia accompanied by hypophosphatemia associated with a malignancy having no skeletal metastases. He described sixty-seven such patients, fifty collected from the literature and seventeen previously unpublished cases from the University Hospitals of Cleveland. The paper made several important observations. First, the author observed that this was not a rare entity. In fact, it was the third most common cause of hypercalcemia at the University Hospitals of Cleveland. Second, he defined the histological group of neoplasms (described earlier) with which the syndrome occurrred. Third, by documenting the reversal of hypercalcemia with surgical resection in twenty patients, he reaffirmed the earlier data (20,61) demonstrating the presence of a humoral mechanism. Last,

he observed that the combination of hypercalcemia and hypophosphatemia was not unlike that seen in patients with primary hyperparathyroidism. As suggested earlier by Albright (59), Lafferty argued for the production of ectopic PTH as the pathogenetic mechanism.

Lafferty's paper provided an impetus that began an earnest search for the humoral factor or factors causing HHM. This quest has come to dominate the literature on the topic. Since 1966, advances in technology have also allowed a more complete description of both the biochemical profile and the process of bone resorption associated with the syndrome. It is worthwhile examining both of these before discussing the proposed pathogenetic factors.

Perhaps the most definitive biochemical characterization of HHM came from Stewart et. al. in 1980 (14). The investigators studied fifty consecutive patients with MAHC. They compared serum calcium levels, serum phosphorus levels, nephrogenous cyclic AMP (NcAMP) excretion, tubular phosphorus thresholds, fasting calcium excretion and plasma 1,25 $(OH)_2$Vit. D concetrations in patients with HHM to those encountered in normocalcemic cancer patients, patients with primary hyperparathyroidism (HPT) and patients with LOH. They found a similar degree of hypercalcemia in patients with HHM, HPT, and LOH. Serum phosphorus levels were low to low normal in patients with HHM and HPT, but were normal in patients with LOH. Likewise, the renal phosphorus threshold was depressed in patients with HHM and HPT and within normal limits in patients with LOH. Fasting calcium excretion was elevated in patients with HHM and in those with LOH. Both groups had markedly higher values than patients with HPT or normocalcemic cancer patients. Patients with HHM and LOH had depressed concentrations of plasma 1,25$(OH)_2$ Vit. D, whereas normocalcemic cancer patients had normal values and patients with HPT had elevated values. Finally,

the most striking finding was the very high levels of NcAMP in patients with HHM. These values averaged fifty percent higher than those seen in patients with HPT,whose values were moderately elevated over those of normocalcemic patients. In distinct contrast, patients with LOH had depressed levels of NcAMP.

The above information was valuable in three respects. First, it painted a biochemical profile of the HHM syndrome, permitting its clinical differentiation from HPT and LOH. Second, it described the biological effects of the putative humoral factor. This factor increases serum calcium, tends to decrease serum phosphorus, decreases renal reabsorption of both calcium and phosphorus, lowers serum $1,25(OH)_2$ Vit. D levels and stimulates production of NcAMP. Third, it provided a biochemical test, NcAMP, that seems to be a marker for HHM. Elevated NcAMP values correlate strongly with the histological tumor types associated with HHM as previously described by Lafferty (12) and others (13,15,20,24).

As in LOH, hypercalcemia in HHM is ultimately caused by increased bone resorption. Evidence from both human (25) and animal studies (21,22) has shown that this occurs as a result of an uncoupling of osteoblast and osteoclast activity. Normally, the activities of osteoblasts and osteoclasts are tightly coordinated so that, despite the continuous and extensive flux of calcium ion between the skeleton and the extracellular fluid, there is no net change in the overall calcium content of either one (5). Likewise, in several metabolic bone diseases such as HPT, secondary hyperparathyroidism associated with renal failure, and Paget's disease, there is increased bone turnover, but both osteoblast and osteoclast activity increase in a coupled fashion. The balance between the two processes is maintained (25,64). In contrast, in HHM the balance is disrupted. Stewart et. al. (25) performed detailed histological and quantitative histomorphometric studies of seven patients with HHM. They found markedly

stimulated osteoclast function, as evidenced by an increased osteoclast surface, in the face of markedly depressed osteoblast function, as evidenced by a reduced osteoblast surface, osteoid surface, and osteoid volume. Hence, it seems that the humoral factor causing HHM is able to uncouple the normal concert between those two bone cells, increasing osteoclastic bone resorption and decreasing osteoblastic bone formation.

The identity of the factor responsible for HHM has sparked much interest. Early reports on HHM (12,59-63) all stressed the biochemical constellation of hypercalcemia and hypophosphatemia. Since these were known effects of parathyroid hormone,Albright (59) and later Lafferty (12) invoked the idea of ectopic PTH production in the tumors as the mechanism for HHM. This concept persisted for some forty years, but it is now well established that native PTH is not the responsible factor. There are three lines of evidence that support this conclusion. First, when the syndrome was more fully characterized biochemically (14), it became obvious that although the clinical syndrome shares some features of HPT, it is not associated with the full range of PTH target organ responses. The factor responsible for HHM mirrors the actions of PTH in causing bone resorption, renal phosphate wasting , and generation of NcAMP (14). However, it lacks the ability to stimulate the conversion of 25-hydroxy-vitamin D to 1,25 dihydroxy-vitamin D and it lacks the ability to stimulate distal tubular calcium reabsorption (14).

The second line of evidence against PTH being the HHM factor comes from immunoassay data. Tashjian, Levine, and Munson first reported the presence of immunoassayable PTH (iPTH) in extracts of six tumors associated with HHM in 1964 (65). Using a quantitative complement fixation assay, Lafferty (12) detected iPTH in an additional 4 cases of HHM. With the advent of

radioimmunoassays (RIA's) for PTH, there were many studies performed that looked for the presence of iPTH in the plasma of patients with HHM (24,66-72). Several of these reports are instructive. Using an RIA system that was specific for the C-terminal region of porcine PTH and which displaced ^{131}I-labelled bovine-PTH, Benson et.al. (69) reported the presence of iPTH in the serum of 95% of 108 unselected patients with HHM (the majority of whom, 60, had HHM). Five of these patients had elevated iPTH values, while the rest had iPTH concentrations in the normal range. The authors argued that in the face of hypercalcemia iPTH values should be suppressed, so that values within the "normal range" actually represented an elevation. Powell et. al. (68) and Stewart et.al. (14) used multiple RIA's with several different antisera directed at regions spanning the entire length of the PTH molecule, including biologically active and inactive native PTH and PTH fragments. Powell and associates were unable to detect iPTH in any of the eleven patients they studied. Stewart et. al. found low-normal to undetectable mean iPTH levels in their HHM population.

Conflicting results such as this led to some argument (69) and a great deal of speculation about the nature of the PTH secreted by the tumors (71) or whether the tumors secreted PTH at all (68). Despite the confusion, two facts became established. First, results varied with different immunoassay systems (5,68,69). Antibodies with specificity for different parts of the PTH molecule seemed to give different results. Second, when iPTH was detected, it was noted that the serum levels did not vary with serum calcium concentrations as would have been expected. Serum iPTH was consistently lower in patients with HHM than it was in patients with HPT (67,69), and although there was a correlation between increasing calcium levels and increasing iPTH levels in HPT, no such correlation was evident in HHM.

These data became more fully interpretable only with the addition of several observations on the nature of native PTH secretion. First, it became evident that serum iPTH is a heterogeneous mixture of both biologically active and inactive fragments (71,73-79). Second, it was noted that there is a nonsuppressible component of iPTH secretion that operates even in the face of hypercalcemia (80). Third, it was discovered that hypercalcemia changes the composition of the circulating iPTH in serum. It increases the proportion of biologically inactive C-terminus PTH fragments and it decreases the proportion of biologically active N-terminus fragments (81,82). These facts suggest an explanation for the disagreement between the studies. By virtue of its low concentration and its lack of correlation with serum calcium levels, the iPTH measured in the patients with HHM most likely represents the nonsuppressible segment of PTH secretion and the accumulation in plasma of biologically inactive C-terminus fragments. The variation seen with different assays was probably based on whether or not they could recognize these biologically inactive fragments. Benson et. al. (69) unknowingly anticipated this conclusion. They pointed out that their high incidence of detecting iPTH relied on their use of a more "sensitive" RIA employing specificity for the C-terminal end of PTH. Further support for this interpretation also comes from Broadus and Stewart (5) and Mallette et. al. (83). Both sets of investigators have recently measured serum iPTH in patients with HHM using a sensitive PTH-RIA with mid-region and C-terminus specificity. Both studies found low concentrations of iPTH that did not correlate with serum calcium concentrations but did correlate with serum creatinine concentrations. This suggests that any iPTH detected in patients with HHM can be explained by retained C-terminal PTH fragments which accumulate following the development of azotemia (84).

The last and most powerful piece of evidence arguing against PTH as the humoral factor in HHM comes from mRNA hybridization data. Simpson et al. (85) used radiolabelled PTH cDNA to probe Northern blots of mRNA extracted from five human tumors and three animal tumors associated with HHM. The authors claimed that they could detect PTH mRNA in as little as 100 ug of control parathyroid tissue using their hybridization scheme. However, they were unable to detect PTH mRNA in as much as 100 mg of tissue from any of the HHM tumors. Therefore, since the probe used in this study contained the coding and flanking sequences for PTH, it is very unlikely that the factor responsible for HHM shares a significant portion of its primary amino acid sequence with PTH or that the HHM-factor is a product of the PTH gene. These results have recently been duplicated by Insogna et. al. in a Leydig cell tumor rat model of HHM (22).

It is evident that HHM is not caused by the ectopic production of PTH. The exact nature of the factor causing this syndrome is not known at present. The leading contenders are prostaglandins, transforming growth factors, and peptide(s) structurally dissimilar to PTH, yet able to stimulate certain PTH receptors. The evidence for each of these possibilities will be discussed separately below.

Prostaglandins

Prostaglandins were first shown to resorb bone in 1970 by Klein and Raisz (40). Using a fetal rat long bone assay (86), these investigators showed that prostaglandins of the E series had potent *in vitro* bone-resorbing activity, similar to that of PTH. This observation prompted speculation that

prostaglandins might play a role in HHM. It is now established that PGE_2 is an important humoral factor in two animal models of HHM.

Work by Tashjian and his group has implicated PGE_2 as the humoral mediator of hypercalcemia in the mouse $HSDM_1$ fibrosarcoma and in the rabbit VX_2 carcinoma. The $HSDM_1$ fibrosarcoma is a transplantable tumor that neither metastasizes to bone nor locally invades bone, but causes hypercalclemia (8,87). The hypercalclemia is reversible with surgical resection of the tumor, indicating a humoral mechanism. Mice bearing this tumor have elevated plasma levels of PGE_2 and its longer-lived metabolite 13,14 dihydro-15-keto-PGE_2 (88-90). It has been shown that $HSDM_1$ cells produce large quantities of PGE_2 (88) and that tumor extracts as well as tumor-conditioned medium contains bone-resorbing activity that can be accounted for quantitatively by the PGE_2 content of the tumor or medium (88,91). Finally, indomethacin, 5,8,11,14-eicosatetraynoic acid, and hydrocortisone, all powerful inhibitors of PGE_2 synthesis in $HSDM_1$ cells, have been shown: 1. to lower plasma concentrations of PGE_2, its metabolites, and serum calcium and 2. to decrease in parallel the PGE_2 content and the bone-resorbing activity of these tumors (88-92).

The Vx_2 carcinoma, also a transplantable tumor, causes hypercalcemia in rabbits. This tumor does not metastasize to bone, but it produces a bone-resorbing factor that causes histomorphometric changes reminiscent of human HHM (91,93). Rabbits harboring this tumor show elevated levels of PGE_2 and its metabolite 13,14-dihydro-15-keto PGE_2 in both plasma and venous drainage of the tumor (94-96). Tashjian et. al. have reported that indomethacin and hydrocortisone prevent the rise in plasma levels of both compounds and reduce the levels of PGE_2 and bone-resorbing activity present in tumor extracts or VX_2 cell culture medium (91,94,95). Furthermore, they claim that continuous

treatment of the rabbits with indomethacin from the time of tumor transplantation prevents the development of hypercalcemia (91,94). Recently, Doppelt et. al. (97) have called these results into question. In sharp contrast to the above results, they observed that on a normal calcium diet, oral indomethacin treatment neither prevented the development of hypercalcemia nor did it lower the serum calcium level once hypercalcemia was present. Also, the investigators were not able to prevent the development of hypercalcemia with dichloromethylene diphosphonate, a potent inhibitor of bone resorption. The only way they were able to reverse the hypercalcemia was to feed the rabbits a calcium-free diet. Hence, despite Tashjian et al's findings, the mechanisms of hypercalcemia in this model are not fully understood. It is likely that intestinal calcium hyperabsorption is the major pathophysiologic feature of the syndrome in these animals.

Despite the compelling evidence for a causitive role in the $HDSM_1$ fibrosarcoma and the suggestion of a contributing role in the VX_2 rabbits, prostaglandins seem to have little if any role as humoral mediators of hypercalcemia in humans (8,9). These compounds were originally proposed to be mediators of HHM in humans based on two facts. It was known that many human tumors produce prostaglandins, PGE_2 in particular (41). Also, there were several early case reports claiming a correlation between hypercalcemia and elevated plasma PGE_2 levels in patients with renal carcinomas (98,99). Seyberth et al. (100) furthered this speculation when they reported elevated levels of 7 *a*-hydroxy-5,11 diketotetranorprostane-1,16 dioic acid (PGE-M), the major urinary metabolity of PGE_2, in twelve of fourteen patients with MAHC. However, seven of fifteen normocalcemic patients with solid tumors also had elevated levels, calling into question the specificity of these findings with regard to

hypercalcemia. Further experience (101-103) has shown that tumor production of prostaglandins does not correlate well with calcium levels and that circulating levels of PGE_2 and/or its metabolites are too low to account for the level of bone resorption necessary to cause hypercalcemia. Finally, despite a few reported successes (103,104), prostaglandin synthesis inhibitors such as indomethacin have not been efficacious in treating MAHC (9). The evidence now suggests that prostaglandins may play a greater role in controlling some local aspects of bone formation or resorption. For example, Minkin et. al. have demonstrated that prostaglandin synthesis inhibitors can suppress the *in vitro* bone resorption caused by systemic factors released by tumors (105). However, the data clearly shows that prostaglandins have no general role as the systemic, humoral bone-resorbing factor causing HHM.

Transforming Growth Factors

In recent years Mundy and his colleagues have proposed the transforming growth factors (TGFs) as possible etiologic agents responsible for HHM. This proposal is based on two observations. First, Tashjian and Levine (106), using the mouse calvarium assay and Raisz et. al. (107), using the fetal rat long-bone assay have demonstrated that epidermal growth factor (EGF) is a potent stimulator of bone resorption in vitro. Second, there is evidence that many human tumors produce TGFs, a family of factors that share biological activities with EGF, including the ability to interact directly with the EGF receptor (48,108-110). Briefly, the characteristics of TGFs include the ability to stimulate cell growth and replication (mitogenesis) and the ability to "transform" normal cell lines (111). "Transformation" is defined as the loss of

density-dependent growth inhibition and development of anchorage-independent growth, detected by the ability to form colonies in soft agar (112). TGF- *a*'s are those which can compete with EGF for binding to its receptor and can independently stimulate anchorage-independent cell growth (113). TGF-ß's, on the other hand, require the presence of EGF to stimulate anchorage-independent growth, and they do not compete with EGF for receptor binding (113). Mundy and his group have presented evidence suggesting pathogenetic roles for both TGF-*a* and TGF-ß in HHM.

The evidence concerning TGF-*a* comes from work done with a Leydig cell tumor. This tumor causes hypercalcemia in rats and has been shown to be a reliable model for HHM (8). Ibbotson et. al. (114) reported that both tumor extracts and conditioned medium from tumor cell cultures contain a bone-resorbing factor that shares certain physical characteristics with TGFs. It is acid stable, sensitive to reducing agents and sensitive to trypsin. They were able to demonstrate co-elution of bone-resorbing activity, mitogenic activity, and soft agar colony-stimulating activity on gel filtration chromatography of tumor extracts and tumor conditioned medium. The gel-filtration fractions containing bone-resorbing activity also contained a factor which is able to compete with labelled EGF for binding to the EGF receptor. Further studies have demonstrated that the addition of EGF receptor antiserum to the fetal rat bone resorption assay inhibits the resorption usually seen with tumor conditioned medium and with EGF, but not that seen with PTH (115). This suggests that bone resorption in the rat Leydig cell tumor model may involve the EGF receptor, supporting the possibility of an etiologic role for TGF-*a*.

The evidence concerning TGF-ß comes from the hypercalcemic variant of the Walker Rat Carcinosarcoma,also shown to be a valid model of HHM (8). As in

the Leydig cell model, D'Souza et. al. found that the bone-resorbing activity present in tumor extracts and tumor-conditioned medium shares certain physical characteristics with TGF,including stability to acid and heat and sensitivity to the reducing agent, dithiothreatol (DTT) (116). They also demonstrated the co-elution of bone-resorbing and soft agar-stimulating activities on gel filtration chromatography (116). However, the TGF activity is different in this model in two respects. It does not seem to inhibit labelled EGF from binding to its receptor and it requires the presence of EGF to cause cell transformation. These observations form the basis for the authors' suggestion that the activity is a TGF-ß.

Evidence for the role of TGFs in human HHM is limited to experience with two tumors, a renal cell carcinoma and a squamous cell lung carcinoma. In both of these tumors, bone-resorbing activity has been shown to have the previously mentioned physical characteristics, stability in acid, sensitivity to DTT, and sensitivity to trypsin, which are also seen TGFs (117,118). Furthermore, Jacobs et. al. have reported that an mRNA isolated from the renal cell tumor and injected into a xenopus ooctye protein translation system stimulates the production of a factor or factors that binds to the EGF receptor (118).

It is impossible to prove categorically whether or not the bone-resorbing and the TGF activities described in these studies are one and the same until these activities are purified to homogeneity. The attempt to accomplish this purification is currently in progress. When and if it is susccessful, it will allow the elucidation of any connections between the bone-resorbing activity, the growth factor activity, and the adenylate cyclase activity (to be discussed next) associated with HHM.

PTH-like Peptides or Adenylate-Cyclase Stimulating Factor(s)

There is growing evidence that tumors associated with HHM produce a peptide or peptides able to specifically interact with selected parathyroid hormone receptors. As reviewed earlier, the evidence against PTH as the pathophysiologic culprit in HHM is strong. Also the preliminary molecular biology data (22,85) makes it unlikely that the HHM factor(s) (HHM-F) physically resembles PTH, at least with respect to its primary protein structure. However, its ability to resorb bone (25) and to stimulate the proximal renal tubular adenylate cyclase system (14) suggests that HHM-F must share some effector mechanisms with PTH. Further support for this notion is supplied by Goltzman and Stewart (119,120), who have demonstrated a third bioactivity held in common between PTH and HHM, one which is not directly related to the clinical syndrome. They showed that serum from patients with HHM contained a factor which could stimulate the PTH-sensitive proximal tubular glucose-6 -phosphate dehydrogenase complex in an *in vitro* cytochemical assay.

Much of the evidence for the existence of biologically active PTH-like peptides in patients with HHM rests on the observation that these patients have elevated levels of NcAMP. It is assumed that these elevated levels result from the ability of HHM-F to stimulate the PTH mediated proximal renal tubular adenylate cyclase system. It is helpful to look more closely at this observation and this assumption. Fifty to sixty percent of the total urinary cAMP content is derived from the renal excretion of plasma cAMP (121,122). The remainder is produced by the kidney, largely in response to circulating PTH, which acts at the proximal tubular adenylate cyclase system (122). Broadus et. al. have shown that the renally produced PTH-sensitive element of urinary cAMP can be quantified by

expressing total urinary cAMP as a function of the glomerular filtration rate and by subtracting the filtered load of cAMP (123) . This expression, known as NcAMP or nephrogenous cyclic-AMP, correlates well with iPTH levels, and its elevation has become a marker for elevated levels of circulating PTH. Several groups have investigated the levels of NcAMP associated with HHM. Kukreja et. al. (124) found elevated NcAMP levels, yet normal iPTH levels, in patients with lung cancer. Rude et. al. (121) reported increased NcAMP levels in a series of patients with MAHC as compared to normocalcemic cancer patients. They found that these elevations were most likely to be found in patients with squamous tumors or renal cell carcinomas (121). Finally Stewart et. al. (14) observed a clear dichotomy between elevated NcAMP levels (similar to patients with HPT) in patients with HHM and depressed levels in patients with LOH. The findings were so striking that the authors argued for a redefinition of the syndrome of HHM based on NcAMP. They suggested that those patients with tumors of expected histology and with elevated NcAMP levels be classified in the HHM group even though they might have limited skeletal metastases.

After having established elevated NcAMP as a reliable marker, investigators began looking for an adenylate cyclase stimulating factor(s) (ACSF) in extracts of tumors associated with HHM. Stewart et. al. first accomplished this in 1983 (120). These investigators employed two *in vitro* systems to probe for PTH-like activity: 1. a canine renal cortical membrane adenylate cyclase assay measuring proximal tubular adenylate cyclase stimulating activity (ACSA) and 2. the PTH cytochemical bioassay mentioned above, which measures PTH-sensitive renal tubular G-6-PD stimulating activity. They demonstrated dose-dependent ACSA and G-6-PD-stimulating activity in four of five tumor HHM extracts tested. Control extracts from tumors of nine

normocalcemic cancer patients and one patient with LOH showed either the absence of such activity or only marginal ACSA (3 patients). PTH-like bioactivity was abolished in both assays in a dose-dependent fashion by the addition to the assay of [Nle ^{8,18}, Tyr ^{34}] bPTH (3-34) amide, a synthetic analogue and specific PTH receptor antagonist (120). This observation strongly suggests that some factor in the extracts was specifically interacting with PTH receptors. However, preincubation of the tumor extracts with PTH antiserum did not alter the bioactivity seen in either assay system, confirming that the stimulation is not due to PTH. Finally, the investigators also demonstrated that the PTH-like bioactivity eluted from gel filtration columns at an apparent molecular weight of 28,000 daltons, a molecular weight much larger than that of PTH.

Since this initial report, tumors from many more patients have been examined for the presence of ACSA. In a recent publication of their laboratory's aggragate experience (125), Stewart et. al. reported the presence of dose-dependent renal cortical ACSA in eighteen of twenty tumors associated with HHM. In contrast only four of thirty-seven control tumors (20 tumors from normocalcemic patients, 7 tumors associated with LOH, 10 nonmalignant tissue samples) were reported to contain any detectable ACSA. In addition, extracts from many of these tumors as well as conditioned medium from cultured tumor lines associated with HHM, have been shown to stimulate PTH-sensitive adenylate cyclase production in clonal osteosarcoma cells (125,126). As in the renal cortical membrane assay, this cAMP production is inhibitable by the PTH receptor antagonist [Nle ^{8,18}, Tyr ^{34}] bPTH (3-34) amide (126).

Investigators in another laboratory have confirmed the pesence of ACSA in tumors associated with HHM (127). Strewler and his associates have

transplanted a cultured human renal cell carcinoma line into nude mice. The mice develop HHM, and conditioned medium for the cell cultures contains ACSA as determined in the renal cortical membrane assay. This ACSA is dose-dependent and inhibitable with the PTH antagonist mentioned previously. Final support comes from animal data. ACSA has been demonstrated in the following models of HHM: a carcinogen induced mouse squamous tumor (21), the Walker rat carcinosarcoma 256 (126), the H-500 rat Leydig cell tumor (126,128), and a naturally occurring canine lymphosarcoma (129).

As established before, HHM is ultimately caused by increased bone resorption (25). The above data is strong circumstantial evidence that this ACSA is associated with HHM. However, to implicate it as the causative factor(s) necessitates showing that it also has bone-resorbing activity. Several groups have recently accomplished this. Klein et. al. have shown that conditioned medium from a human renal cell tumor line causes bone resorption in the fetal rat long bone assay (130). Furthermore, both Klein et. al. (130) and Stewart et. al. (125) have demonstrated co-purification of ACSA and bone-resorbing activity in initial partial purification schemes. Klein et. al. observed co-elution of these activities from a gel filtration column, which achieved a 20-fold purification of the ACSA. Stewart et. al. have purified the ACSA from tumor extracts much further (some 4800-fold) using reverse phase HPLC and have still obseved potent bone-resorbing activity associated with the ACSA. Similar observations have been made in two of the animal tumor models of HHM shown to possess ACSA. Conditioned medium from the Walker rat 256 carcinosarcoma has been shown to possess bone-resorbing activity (126), and both bone-resorbing activity and ACSA have been shown to co-migrate on gel filtration of conditioned medium from Rice H-500 rat Leydig cell tumor lines (128).

As with the TGF activity, until the ACSA in purified to homogeneity, it will be impossible to ascertain whether the cAMP-generating activity and the bone-resorbing activity reside in the same molecule. However, the data gathered thus far seem to anticipate this result.

Final support for the involvement of an adenylate cyclase-stimulating factor(s) in HHM comes from recent work by Merendino and Stewart. Since many tumors causing HHM are of squamous epithelial origins (14), these investigators tested for the presence of ACSA in culture medium from non-malignant human keratinocytes. They demonstrated that,indeed, these cells produced a factor or factors with dose-dependant ACSA in the rat osteosarcoma cell assay (132). Furthermore, this activity is inhibitable by the PTH receptor antagonist, [Nle ^{8,18},Tyr^{34}] bPTH (3-34) amide, in a fashion similar to the ACSA associated with HHM tumor lines. Subsequent experiments have shown that this factor or factors also possesses bone-resorbing activity in the fetal rat long bone assay (Stewart, unpublished observations). Currently, it is not known whether it is by chance or by design that a normal keratinocyte product has the ability to interact with PTH receptors and to cause bone resorption. However, it suggests that the non-regulated production of such a factor(s) by malignant squamous epithelial cells and its release into the general circulation may be a pathogenetic event in HHM.

Partial purification of this ACSA has allowed the elucidation of several of its physical characteristics. First, it is a protein. Several studies have shown the loss of ACSA and bone-resorbing activity when samples were treated with trypsin (125-128,130). Gel filtration chromatography suggests that it has an apparent molecular weight of approximately 30,000 daltons (21,22,130,131). However, based on SDS PAGE results, a molecular weight of 15,000 seems more likely (Burtis W and Stewart A, unpublished observations). Finally, the protein,

at least in its partially purified form, has been shown to be stable under conditions of dilute acid, freezing and thawing, lyophilization, and boiling at 100°C for up to fifteen minutes (120)

It was in the setting of attempts to isolate and purify the ACSF discussed above that the studies to be described shortly were undertaken. They are a series of experiments exploring different physical characteristics of partially purified ACSF. The behavior of ACSF was examined under oxidizing and reducing conditions, and at different pH's. It was subjected to cyanogen bromide digestion in an attempt to generate an active fragment. Finally, the possibility that it might be a glycoprotein was explored. The goals of these experiments were three-fold: 1. to characterize the molecule(s) more fully, 2. to provide guidelines on handling the increasingly pure protein, and 3. to compare its physical characteristics to those reported for the TGF activity associated with HHM.

MATERIALS AND METHODS

Adenylate Cyclase Assay

PTH-like activity was measured using a modified version of the canine renal cortical membrane adenylate cyclase assay described by Nissenson et. al. (133). The assay measures the conversion of ^{32}P-ATP to ^{32}P-cAMP triggered by the stimulation of a PTH receptor-mediated adenylate cyclase system contained in a renal membrane preparation. Purified renal cortical membranes were prepared as follows. Cortical tissue was dissected from a freshly removed canine kidney and homogenized. Membranes were separated from other cellular elements by sucrose density centrifugations, after which they were resuspended in 0.25 M sucrose, 10mM EDTA and 5.0 mM Tris-HCl, pH 7.5 at a protein concentration of 3.2 mg/ml. The membranes were stored in aliquots at -80°C until used in the assay.

The reaction mixture or "cocktail" consists of 60 microliters per tube of 50 mM Tris-Hcl (pH 7.4), 5 mM Mg Cl_2, 10 mM KCl, 1 mg/ml BSA, 1 mM EGTA, 2 mM DTT, 0.11 µg/ml creatine kinase, 5 mM creatine phosphate, 1 mM unlabelled cAMP, 10 µM 5' guanylyl imidodiphosphate, 6 µg canine renal cortical membrane protein and approximately 500,000 cpm ^{32}P-ATP. The creatine phosphate and creatine kinase constitute an ATP regeneration system, and the unlabelled cAMP is added to retard degradation of the newly formed ^{32}P-cAMP by phosphodiesterase. 5' guanylyl imidodiphosphate is a hydrolysis-resistant GTP analogue which has been shown to increase greatly the sensitivity of adenylate cyclase to PTH (134,135). The reactions were initiated by addition of 10

microliters of bPTH (I-34) [Beckman Bioproducts, Palo Alto, CA] as a standard, 10 microliters of 5 mM acetic acid/ 0.5 mg/ml BSA (HAc/BSA) as a basal, or 10 microliters of experimental sample, all added at 0°C. The reactions were allowed to proceed at 30°C for 30 minutes and were then terminated with the addition, at 0°C, of 100 microliters of 2 mM ATP, 0.5 mM cAMP, and 10,000 cpm ^{3}HcAMP as a recovery standard. Reaction tubes were then boiled for 3 minutes at 100°C, diluted with 800 microliters of water and centrifuged at 3000 X g for 10 minutes.

^{32}P-cAMP generated in the reactions was separated from unused ^{32}P-ATP using the chromatographic method of Salomon et. al. (136). The reaction mixture (1 ml.) was applied to Dowex AG 50 W-X4 (200-400 mesh) cation exchange columns and eluted with water onto Alumina columns. The cAMP was then eluted from the alumina with 5 mls. of 0.1 M Imidazole and collected in scintillation vials containing 15 mls of Optiflour liquid scintillation fluid (Packard). Radioactivity was measured with a dual isotope ^{3}H/^{32}P program and all values were corrected for ^{3}H cAMP recovery. Results are expressed as the percentage increment of ^{32}P counts over basal activity.

Source of ACSF

The adenylate cyclase stimulating factor(s) or ACSF used in these experiments was extracted from a breast carcinoma. The patient was defined as having HHM by virtue of an elevated serum calcium (12.9 mg%, nl. 9.5-10.6), absence of any skeletal metastases on bone scan or at autopsy, elevated nephrogenous cAMP (4.66 nmol/dl GF, nl .5-2.5) and normal commerical

immunoreactive PTH and plasma $1,25(OH)_2$ vitamin D levels. Four and one half grams of tumor tissue obtained at autopsy was subjected to a three step purification scheme. First, it was extracted with acid/urea as previously reported (120). Briefly, tissue was homogenized in a Waring blender in 0.1 M Tris HCl, pH 7.4 at 0°C and then centrifuged for 15 minutes at 27,000 X g. The pellet was then extracted in 8 M urea, 0.2 M HCl and 0.1 M cysteine for 1 hour. The resulting extract was centrifuged for 15 minutes at 27,000 X g and the supernatant was dialyzed against 12 liters of water over 24 hours. The dialysate was subsequently divided into aliquots and lyophilized. The second step was an ethanol-sodium chloride extraction (125). The lyophilized acid/urea extract was resuspended in 10 mM acetic acid containing 100 mM NaCl. Ice cold ethanol was added to a final concentration of 20% (vol:vol). This mixture was kept on ice for 15 minutes, being occasionally vortexed, and was then centrifuged at 16,000 X g for 15 minutes. The supernatant was removed, frozen on dry ice, and lyophilized. The third and final step in the partial purification consisted of reverse phase HPLC. Samples of the ethanol-NaCl extract were suspended in 0.1% trifluoroacetic acid and were applied to a Vydac C_{18} reverse phase column (Separation group, Hesperia, CA). Using a Waters Associates (Milford, MA) model 680 controller, U6-K injector, two 510 pumps and a 440 detector, the column was exposed to a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml per minute. The partially purified ACSF represents five pooled 2-ml fractions collected from 30% to 36% acetonitrile. These fractions corresponded to the only HPLC fractions with significant cyclase stimulating and bone resorbing activity (125). The pooled fractions were divided into 20 microliter aliquots, frozen on dry ice, lyophilized, and stored at -70°C until used in experiments. These pooled HPLC fractions were

shown to have a specific activity of 12,000 nanogram equivalents (ng.eq.) of bPTH (I-34)/ml in the renal adenylate cyclase assay. This represents approximately a 4800-fold purification over the crude acid/urea extracts described previously (120,125,137).

Oxidation and Reduction Experiments

Bovine-PTH(I-34) and partially purified ACSF were oxidized with hydrogen peroxide for varying lengths of time. Sample was combined with hydrogen peroxide at final concentrations of 200 ng/ml of PTH (I-34) or 2.4 ng.eq./ml ACSF and 0.044 M H_2O_2, respectively. Reactions were allowed to proceed at 25°C for thirty minutes, one, two, or three hours and were terminated by freezing on dry ice, and lyophilizing. The samples were resuspended at their initial concentrations in HAc/BSA after which they were assayed for cAMP generating activity.

PTH (I-34) and partially purified ACSF were exposed to three different reducing agents: *B*-mercaptoethanol (*B*-ME), Dithiothreitol (DTT), and L-cysteine. Samples were mixed with reducing agents at final concentrations of 200 ng/ml PTH (I-34) or 2.4 ng.eq.PTH/ml of ACSF and 300 mM *B*-ME, 65 mM DTT, or 130 mM L-cysteine. The reaction was carried out at 25°C for 17 hours, after which time the samples were placed at 0°C and assayed in the renal cAMP assay.

Partially purified ACSF was also sequentially oxidized and reduced. Samples of HHM-F were exposed to 0.044 M H_2O_2 for three hours at 25°C, frozen on dry ice and lyophilized as described above. Samples were then resuspended in HAc/BSA and then mixed with reducing agents to give final concentrations of 300

mM βME, 65 mM DTT, or 130 mM L-cysteine and 2.4 ng.eq. PTH/ml of ACSF. These reaction mixtures were incubated at 25°C for 20 hours and then assayed for cAMP generating activity in the renal adenylate cyclase assay.

pH Experiments

PTH and partially purified ACSF were exposed to various pH's ranging from 3 to 10 in an ammonium acetate buffer system. Ammonium acetate was chosen for its volatility. Aliquots of either ACSF or PTH in HAc/BSA were lyophilized and then resuspended in 100 mM ammonium acetate previously adjusted to the desired pH with either glacial acetic acid or concentrated ammonium hydroxide. They were incubated at room temperature for 2 hours. One half of the samples were then frozen on dry ice, lyophilized for a second time, resuspended in HAc/BSA, and immediately assayed for cAMP generating activity. The remainder of the samples were simply assayed for cAMP generating activity, with and without dilutions, at the completion of their 2-hour incubation in 100 mM ammonium acetate. Controls were treated in an identical fashion except that HAc/BSA was substituted for the pH-adjusted ammonium acetate. 100 mM ammonium acetate adjusted to various pH's, but without HHM-F or PTH as agonist, was also run in the renal cyclase assay as a control.

Cyanogen Bromide Experiments

PTH or partially purified ACSF was exposed to cyanogen bromide (CNBr) digestion. Sample was mixed with either a twenty-five or fifty-fold excess (mole/mole) of CNBr dissolved in 70% formic acid and incubated at room temperature for four hours. The reaction was terminated by the addition of ten volumes of de-ionized water at 0°C and freezing on dry ice. The digests were then lyophilized, resuspended in HAc/BSA and assayed for cyclase stimulating activity. One of the digests so prepared was also run over reverse phase HPLC on a Vydac C_{18} column with a buffer gradient of 25%-35% acetonitrile in 0.1% trifluoroacetic acid at a rate of one ml./min. Two ml. fractions were collected, lyophilized, resuspended in HAc/BSA and assayed for cyclase stimulating activity.

Lectin Affinity Columns

Lectin affinity columns were constructed as follows. Plastic tuberculin syringes were fitted with a pourous polystyrene plug. 0.03 cc of either Concanavalin-A (Con-A) or wheat germ lectin (WGL) covalently bonded to Sepharose beads (Con-A-Sepharose, WGL-Sepharose, Sigma, St. Louis, Mo.) was placed on top of the polystyrene. Samples were subsequently added to the open end of the syringe, and were eluted with the aid of a brief spin in a table-top centrifuge.

Loading buffer for the Con-A columns consisted of 125mM sodium chloride, 12.5mM ammonium acetate, 1mM calcium chloride and 1mM manganese chloride adjusted to a pH of 6.0. Con-A eluting buffer consisted of the loading buffer plus 500mM *a* -D-methyl-mannoside. Loading buffer for the WGL column

consisted of 125mM sodium chloride and 12.5mM ammonium acetate adjusted to a pH of 7.0. WGL eluting buffer consisted of the loading buffer plus 0.2 M N-acetyl-D-glucosamine.

Con-A binding experiments were performed in the following fashion. First, columns were washed with 5 mls of loading buffer, after which sample was added. ^{125}I-labelled *B* - HCG (kindly provided by the Gyn. Endocrinology Laboratory, YNHH) was used as a positive control. A 100 ul aliquot of loading buffer containing 0.66 ng. of ^{125}I-*B* -HCG having 100,000 cpm was applied to the column. Fifteen minutes was allowed for binding and the loading buffer was eluted and collected. The column was washed seven times with 100 ul of loading buffer; each wash was collected and saved. Seven eluting steps were then performed: 100 ul aliquots of eluting buffer were added to the column, were allowed to remain for fifteen minutes, and were eluted and saved. All fifteen collected fractions (one loading sample, seven washes, seven elutions) were then tested for the presence of ^{125}I-*B* -HCG in a gamma counter. The column itself was also counted so that recoveries could be calculated. The above experiment was run in an identical fashion with partially purified ACSF. A 100 ul sample , containing 0.72 ng.eq. PTH of ACSF suspended in Con-A loading buffer, was applied to the column. The sample loading buffer, seven washes, and seven elutions were collected as before and were assayed for bioactivity in the renal adenylate cyclase assay.

A similar preliminary experiment was performed with a wheat germ lectin column. A 0.7 ng.eq.PTH sample of ACSF, suspended in 50 ul of WGL loading buffer, was applied to the column. Fifteen minutes was allowed for binding to occur and then the sample buffer was eluted and saved. The column was washed twice with 50 ul of loading buffer, the fractions being collected and saved.

Following these washes, a total of three elution steps were performed: 50 ul of elution buffer was added, allowed to remain for fifteen minutes, and then eluted and saved. Finally, the sample loading buffer, the two washes, and the three elutions were assayed for adenylate cyclase-stimulating activity in the renal adenylate cyclase assay.

RESULTS

Renal Cortical Adenylate Cyclase Assay Standard Curves

Figure 1 shows the dose-response curves of synthetic bPTH (I-34) in the guanyl-nucleotide amplified canine renal cortical adenylate cyclase assay (cyclase assay). This curve represents the averaged results from all the individual assays performed. As reported previously (120,125-128,137), this dose-response curve demonstrates that the renal adenylate cyclase assay has a minimum concentration detection limit of approximately 200 pg PTH/ml. It is linear over the range of 0.2 ng PTH/ml. to 20 ng. PTH/ml., and it reaches a maximum stimulation of approximately five and one half times the basal activity at a concentration of PTH between 20 ng./ml. and 200 ng./ml. Figure 1 also shows the averaged dose-response curve of the pooled HPLC - ACSF preparations described previously. As can be seen, this curve is nearly identical to that of bPTH(1-34). At the concentrations assayed, the curves are essentially linear. Using these curves it is possible to extrapolate and express the HHM-derived ACSF in PTH equivalents. Thus, a forty-fold dilution of partially purified ACSF (representing one of the 20 *u*l aliquots described in the Methods section diluted out to 800 *u*l) can be shown to have an average ACSF concentration of 2.3 ng.eq.PTH/ml.

Oxidation-Reduction Experiments

In order to ascertain whether the PTH-like bioactivity could be recovered from polyacrylamide gels run under reducing conditions, and in order to compare it to TGFs, ACSF was exposed to reducing agents. Figure 2 shows dose response

curves for both PTH and ACSF assayed alone or in the presence of reducing agents as described in the Methods section. As can be seen from these figures, at the two most concentrated dilutions, DTT seems to inhibit the cyclase assay mildly. At these same dilutions L-cysteine profoundly suppresses cAMP formation. However, when the reaction mixtures are assayed at greater dilutions, the curves converge. This demonstrates that when the assay-inhibiting effects of the reducing agents are diluted out, neither PTH (Fig. 2a) nor ACSF (Fig. 2b) is affected by the exposure to reducing conditions. Table 1 displays mean stimulation from four cyclase assay runs of control and reduced ACSF. Again, at concentrations in which DTT and L-cysteine do not affect the cyclase assay, the bioactivity of the samples exposed to reducing agents is identical to that seen with unreduced ACSF.

TABLE 1

	ACSF DILUTIONS			
	1 : 40	1 : 80	1 : 160	1 : 320
CONTROL	3.316 ± 0.427	2.90 ± 0.402	2.41± 0.390	1.82 ± 0.196
B -ME	3.651± 0.317	3.08 ± 0.405	2.47 ± 0.399	1.99 ± 0.364
DTT	2.60 ± 0.055	2.28 ± 0.178	2.00 ± 0.366	1.76 ± 0.215
L-CYS	0.035 ± 0.008	1.35 ± 0.395	2.11± 0.273	1.81 ± 0.271

It was known that PTH loses its bioactivity when oxidized (139). For this reason both PTH and the partially purified ACSF were exposed to hydrogen peroxide. In contrast to the reducing agent experiments, it was found that both of these molecules are sensitive to oxidation. Figure 3 shows the dose-response

curves generated by PTH and ACSF after exposure to hydrogen peroxide (H_2O_2) for varying lengths of time. PTH bioactivity is almost completely destroyed by exposure to H_2O_2 for as little as thirty minutes (Fig. 3a). ACSF is less sensitive to this oxidant. Residual cyclase activity is present even after 3 hours exposure to H_2O_2 (Fig.3b). Figure 3c demonstrates the time courses for the progressive loss of ACSF and PTH bioactivity upon exposure to H_2O_2. Again, it is seen that ACSF is oxidized much more gradually than is PTH.

Finally, an attempt was made to reverse the loss of bioactivity seen with oxidation by exposing the H_2O_2-treated ACSF to reducing conditions. Figure 4 shows the results of this sequential oxidation and then reduction. The effect of oxidation is not reversed by any of the three reducing conditions employed.

pH Experiments

In an attempt to determine whether exposure to different pH's affected its bioactivity, the ACSF was incubated in 100 mM ammonium acetate adjusted to various pH's, as described above. These experiments were prompted by the poor recoveries of bioactivity from ion-exchange columns used in another series of experiments with ACSF. Preliminary studies had suggested that the neutral pH's involved in the attempts at ion-exchange chromatography might be affecting the ACSF (Stewart, unpublished data). Ammonium acetate was the buffer used for the ion-exchange experiments. Because of this, and because it is volatile, ammonium acetate was also chosen as the buffer in these experiments. Figure 5a shows the effects of this buffer system adjusted to pH's from 3 to 10 on basal activity in the renal cortical adenylate cyclase assay. There is a marked inhibition of basal activity in the assay below a pH of 6. From pH's 6-9 the basal

activity appears to be appropriate, while at pH 10 there is a marked stimulation of basal adenylate cyclase activity.

Figure 5b displays the results of two typical experiments in which either PTH or ACSF was incubated at various pH's. In these experiments, the agonists were added directly to the cyclase assay, still dissolved in the pH-adjusted ammonium acetate buffer. As expected from the prior experiment, there was profound depression of adenylate cyclase stimulation at pH's 4 and below. At pH values 6-9, there was moderate (35 -40 %) loss of bioactivity as compared to controls. At a pH of 10, both bPTH and ACSF displayed apparent stimulation as compared to that seen at the other pH points. With ACSF, pH 10 gave stimulation above that shown by the control. Both of these curves are similar in shape to the curve in Figure 5a.

Figure 5c also displays profiles of adenylate cyclase stimulation generated by PTH or ACSF after exposure to various pH values. However, in these experiments, the ammonium acetate was lyophilized and the sample was resuspended in the usual vehicle (HAc/BSA) prior to assay. When treated in this manner, there was little loss of activity for either PTH or ACSF as compared to controls. Bioactivity at each pH value was over 90% of control activity with PTH. ACSF bioactivity was greater than 95% at all except one of the pH points tested. At a pH of 10 there was a loss of ACSF bioactivity.

CNBr Digest

ACSF was subjected to cyanogen bromide (CNBr) digestion in an attempt to produce a peptide fragment that retained biological activity. PTH served as a positive control. When bPTH was exposed to a 50-fold excess of CNBr (as described in methods) all adenylate cyclase stimulating activity was destroyed.

ACSF was incubated with both a 25 and 50-fold molar excess of CNBr. The resulting digests had residual bioactivity, representing 11% and 13%, respectively, of the undigested control bioactivity (in ng.eq.PTH/ml). In order to ascertain whether this residual bioactivity was due to undigested ACSF or to an active fragment, the digests were chromatographed using reverse phase HPLC as described in the Methods section under "Source of ACSF" . When this was done, the residual bioactivity eluted at 32% acetonitrile, the precise position in the gradient at which ACSF,not exposed to CNBr, also elutes.

Lectin Experiments

In order to determine whether ACSF might be a glycoprotein and also to evaluate the use of lectins as a purification strategy, the partially purified ACSF was applied to both Concanavalin A and Wheat Germ lectin columns as described in methods. ^{1,25}I-labelled ß-HCG was used as a positive control. As can be seen in figure 6-a, this glycoprotein specifically binds to a concanavalin-A sepharose column and is selectively eluted with good recovery from the column with *a*-methyl-mannoside, the specific eluting agent. However, when ACSF was applied to the columns in a similar fashion (figure 6b, 6c) bioactivity was recovered only in the buffer eluted from the loading step. There was no bioactivity in the elution fractions and overall there was a very poor recovery of the total bioactivity (<20% for concanavalin A, <10% for wheat germ lectin) applied to the columns.

Figure 1

Renal adenylate cyclase stimulating activity given by partially purified preparations of ACSF from a human breast carcinoma and by bovine1-34 parathyroid hormone. Stimulation of the assay is expressed by the ratio of ^{32}P-cAMP generated by a sample to ^{32}P-cAMP generated by a basal or blank run. These curves represent the aggregate dose-response curves averaged over each individual assay performed.

FIG. 1

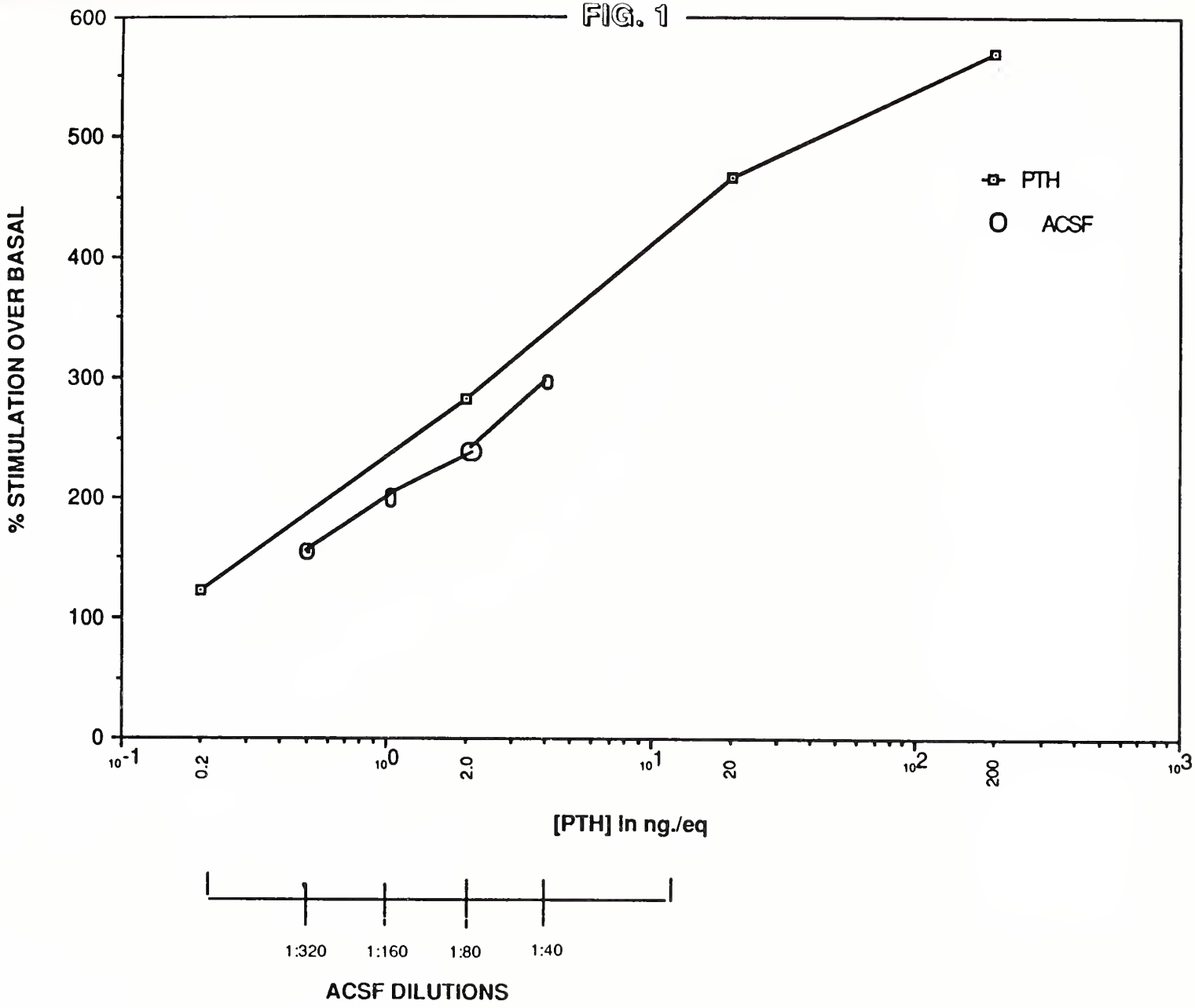

600
500
400
300
200
100
0
% STIMULATION OVER BASAL
PTH
ACSF
10^{-1}
0.2
10^{0}
2.0
10^{1}
20
10^{2}
200
10^{3}
[PTH] In ng./eq
1:320
1:160
1:80
1:40
ACSF DILUTIONS

Figure 2

Results of a typical reduction experiment. Curves represent the dose-response curves generated by dilutions of the reaction mixtures described in Methods. Fig. 2a gives the results for bPTH(1-34); Fig. 2b gives the results for ACSF. Symbols identify curves representing either control PTH, control ACSF, or one of the reducing conditions as described in Methods.

FIG 2a

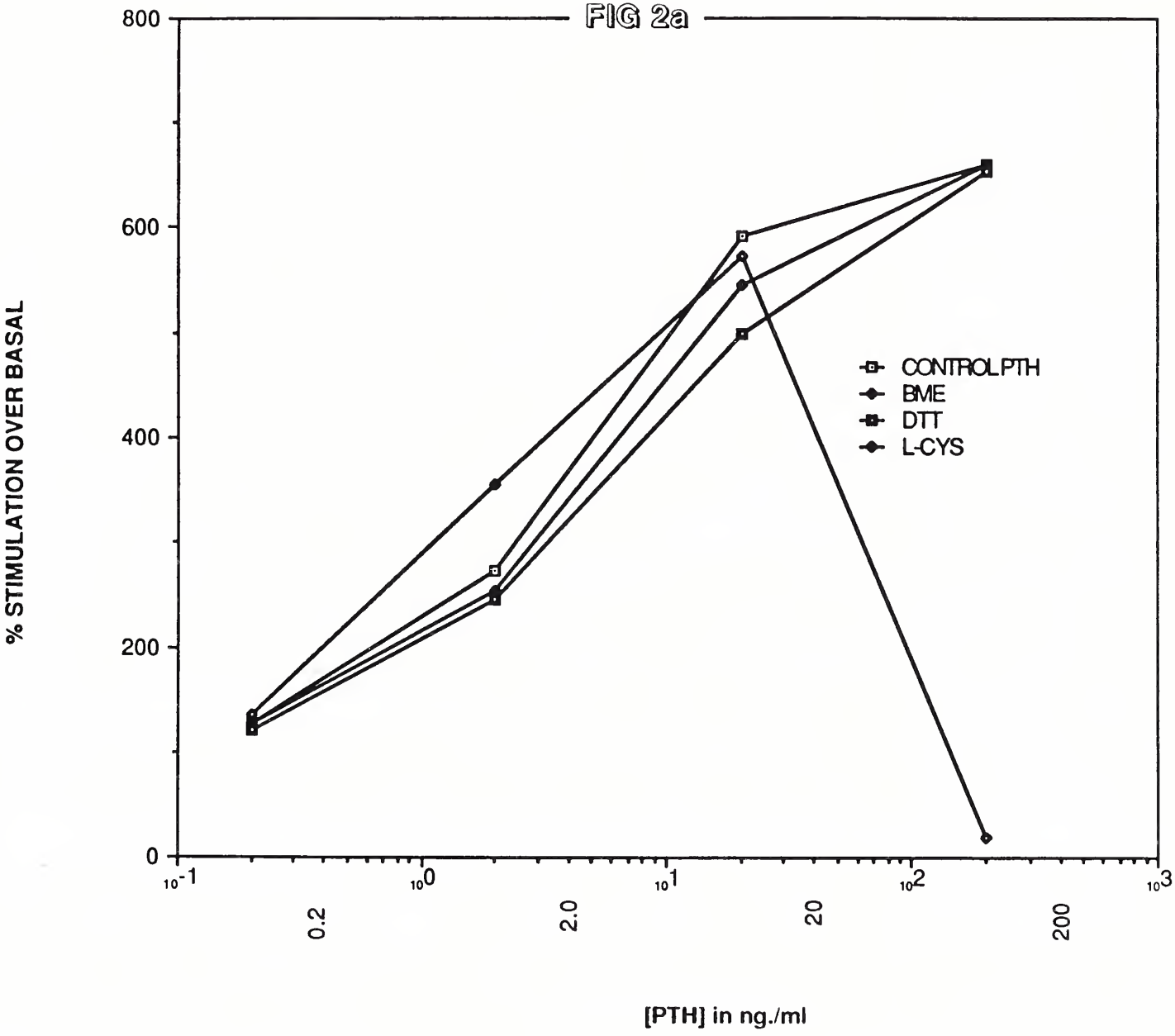

800
600
400
200
0
% STIMULATION OVER BASAL
CONTROL PTH
BME
DTT
L-CYS
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
0.2
2.0
20
200
[PTH] in ng./ml

FIG. 2b

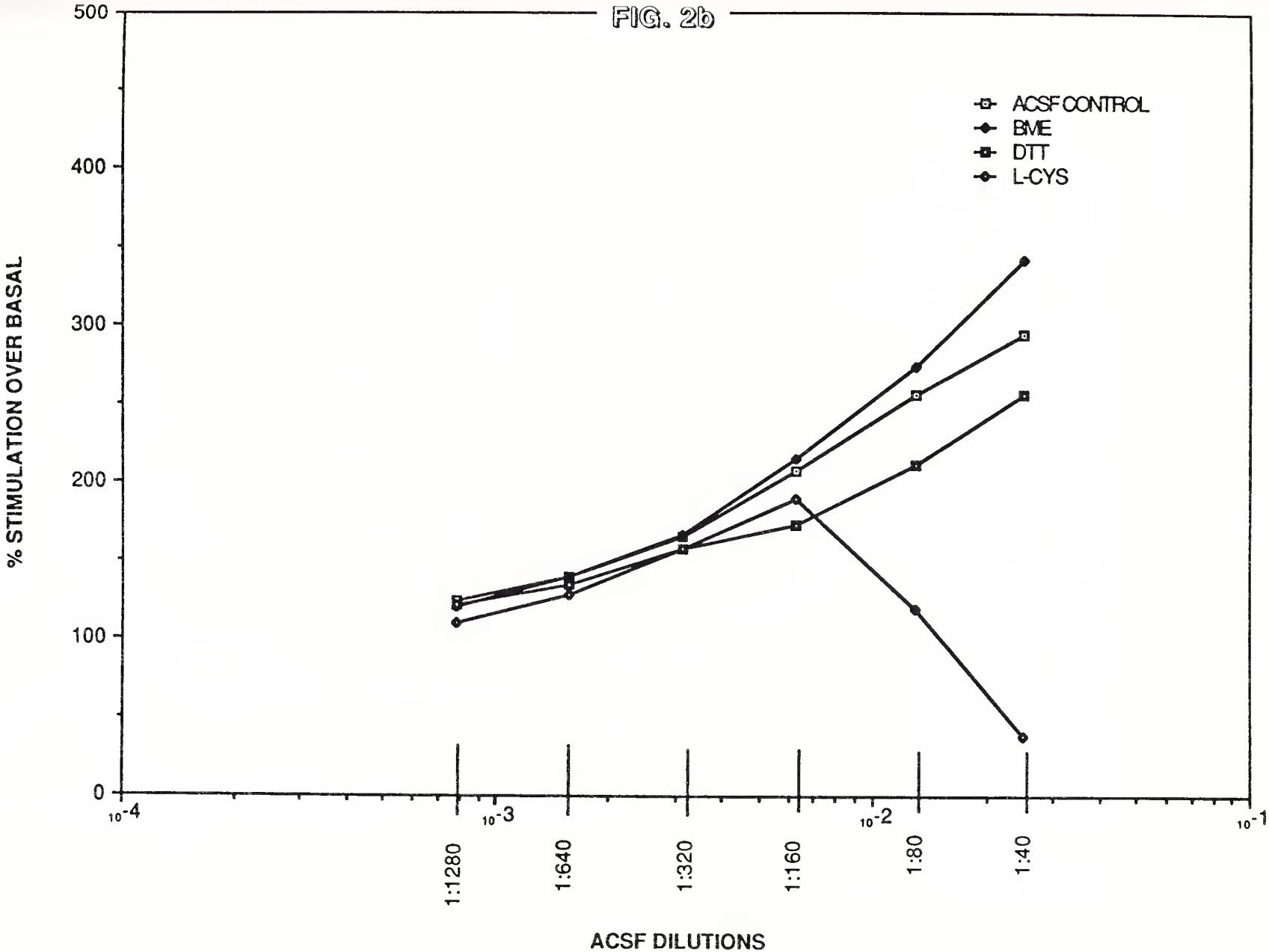

500
400
300
200
100
0
% STIMULATION OVER BASAL
ACSF CONTROL
BME
DTT
L-CYS
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1:1280
1:640
1:320
1:160
1:80
1:40
ACSF DILUTIONS

Figure 3

Results of oxidation experiments with bPTH(1-34) and ACSF. Curves represent the dose-response curves generated by dilutions of the reaction mixtures described in methods. Each curve represents either PTH or ACSF oxidized for a different length of time. Figure 3c shows the loss of bioactivity of both PTH and ACSF with progressively longer periods of oxidation. Note the more gradual loss of bioactivity seen with ACSF.

FIG. 3a

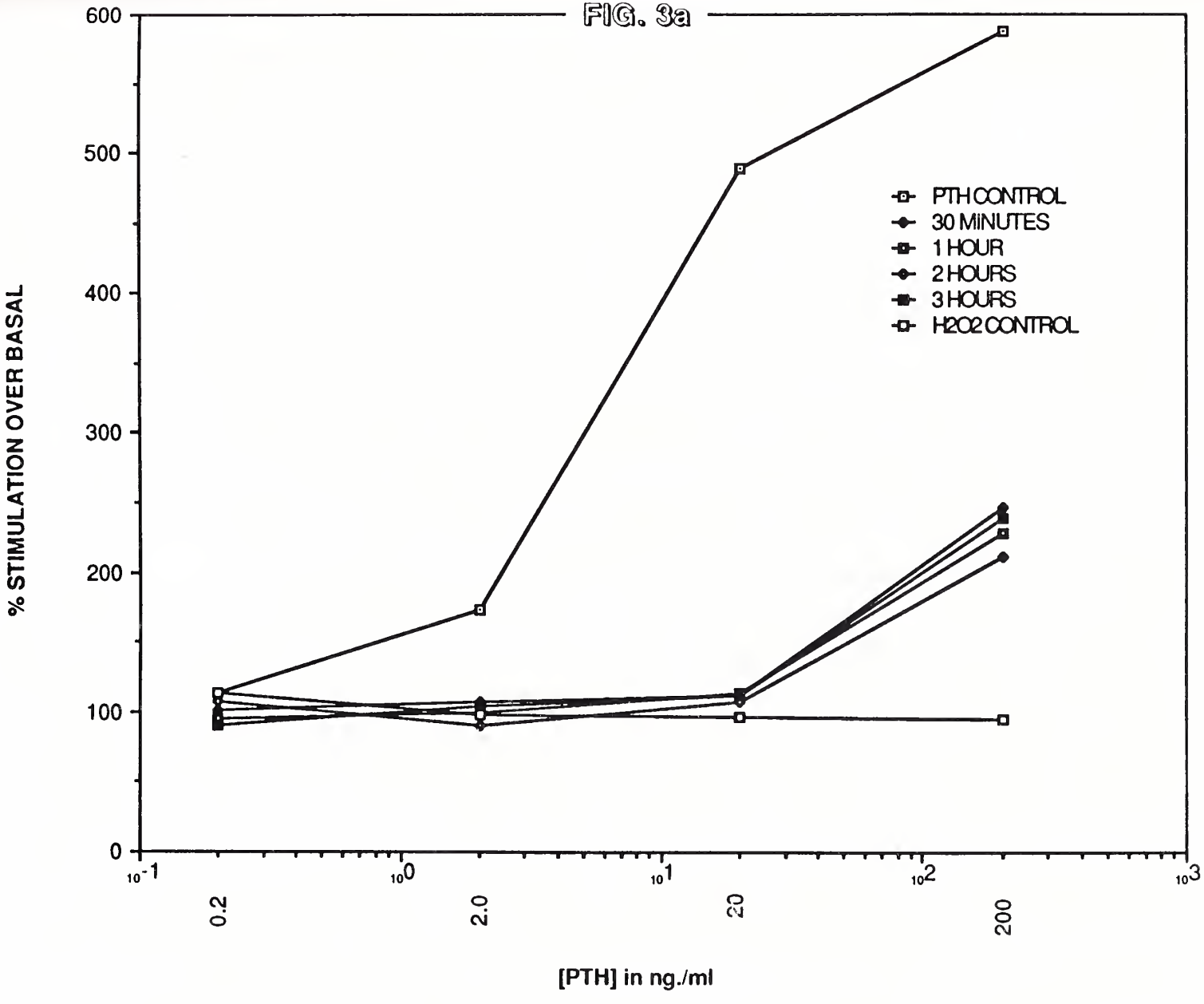

600
500
400
300
200
100
0
% STIMULATION OVER BASAL
PTH CONTROL
30 MINUTES
1 HOUR
2 HOURS
3 HOURS
H2O2 CONTROL
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
0.2
2.0
20
200
[PTH] in ng./ml

FIG. 3b

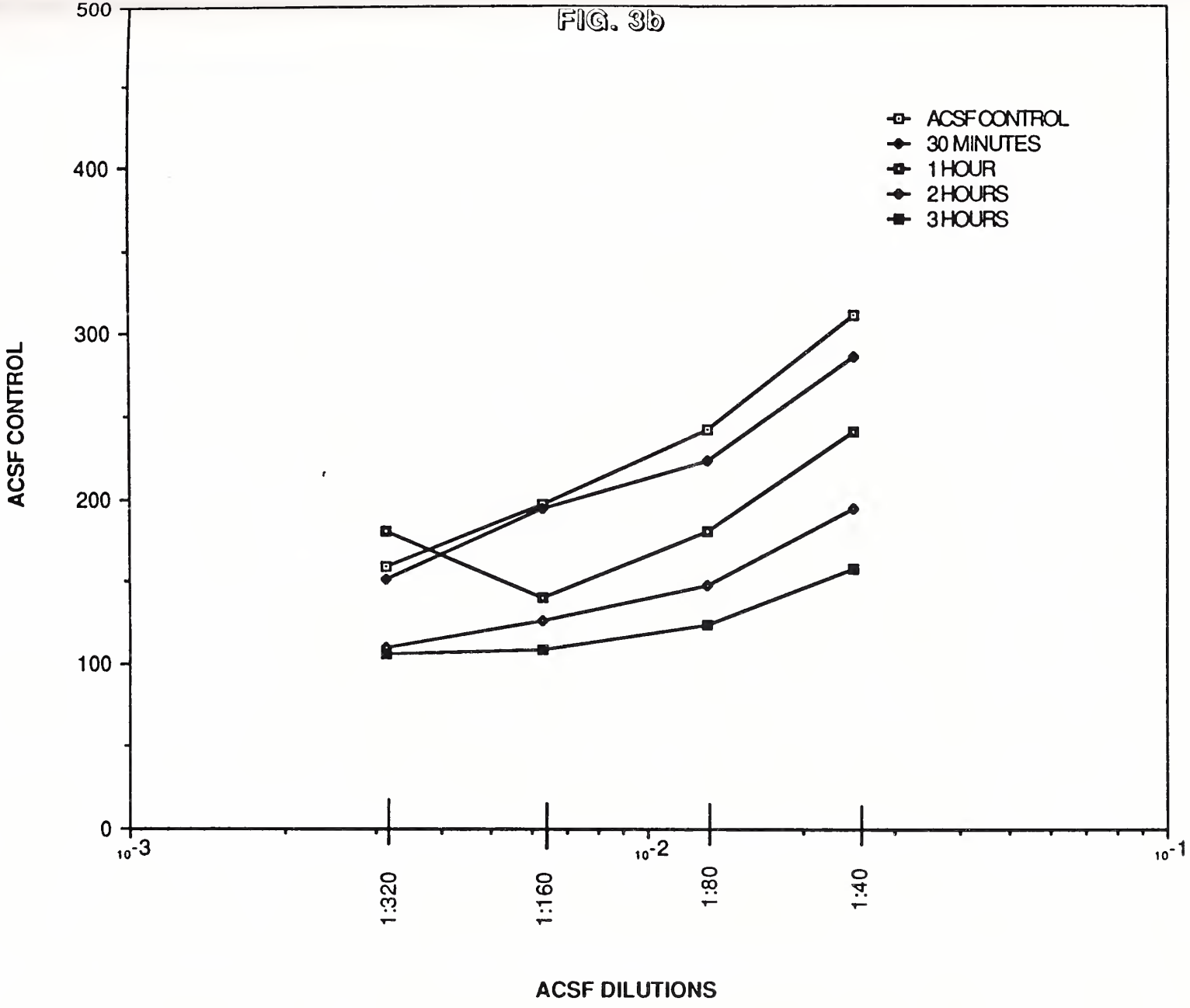

500
400
300
200
100
0
ACSF CONTROL
ACSF CONTROL
30 MINUTES
1 HOUR
2 HOURS
3 HOURS
10^{-3}
10^{-2}
10^{-1}
1:320
1:160
1:80
1:40
ACSF DILUTIONS

FIG. 3c

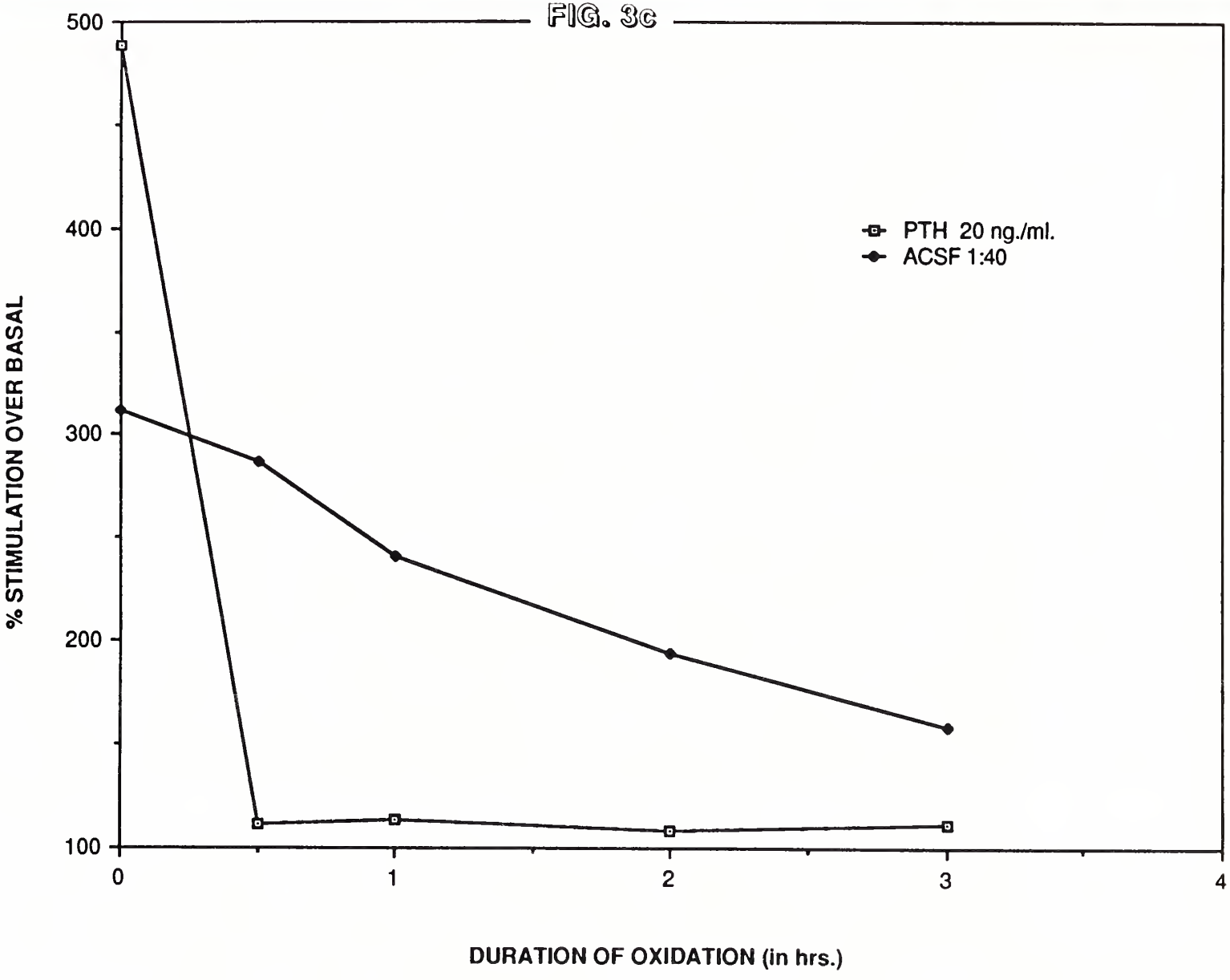

500
400
300
200
100
0
1
2
3
4
% STIMULATION OVER BASAL
PTH 20 ng./ml.
ACSF 1:40
DURATION OF OXIDATION (in hrs.)

Figure 4

Results of an experiment in which ACSF was oxidized and then subsequently reduced. As identified in the legend, curves represent the dose-response curves generated by control ACSF, ACSF oxidized for three hours in H_2O_2, and ACSF oxidized for three hours and subsequently reduced as before with BME, DTT, or L-CYS.

FIG. 4

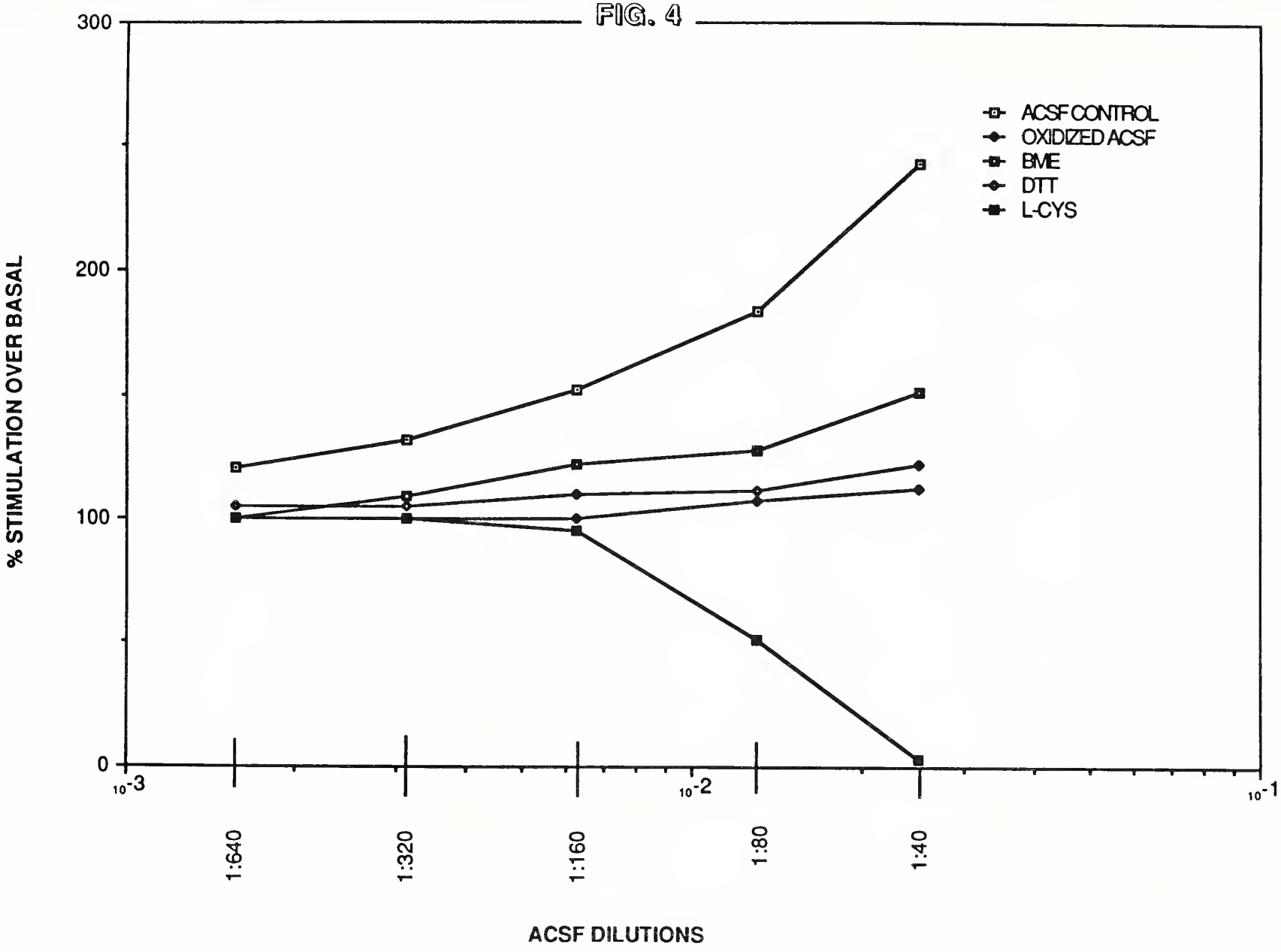

300
200
100
0
% STIMULATION OVER BASAL
ACSF CONTROL
OXIDIZED ACSF
BME
DTT
L-CYS
10^{-3}
10^{-2}
10^{-1}
1:640
1:320
1:160
1:80
1:40
ACSF DILUTIONS

Figure 5

Results of pH experiments with bPTH (1-34) and ACSF.
- 5a displays the effects of 100mM ammonium acetate at different pH's on the renal cyclase assay.

- 5b represents the profile of bioactivity of PTH and ACSF over various pH's. In these experiments the sample was added to the cyclase assay dissolved in the pH-adjusted ammonium acetate. Controls are equivalent amounts of ACSF or PTH assayed in the standard fashion as described in Methods.

- 5c also represents the profile of bioactivity of PTH and ACSF over various pH's. However, in these experiments the ammonium acetate was lyophilized and the samples were resuspended in 5mM acetic acid / 0.5 mg/ml BSA immediately prior to assay.

FIG. 5a

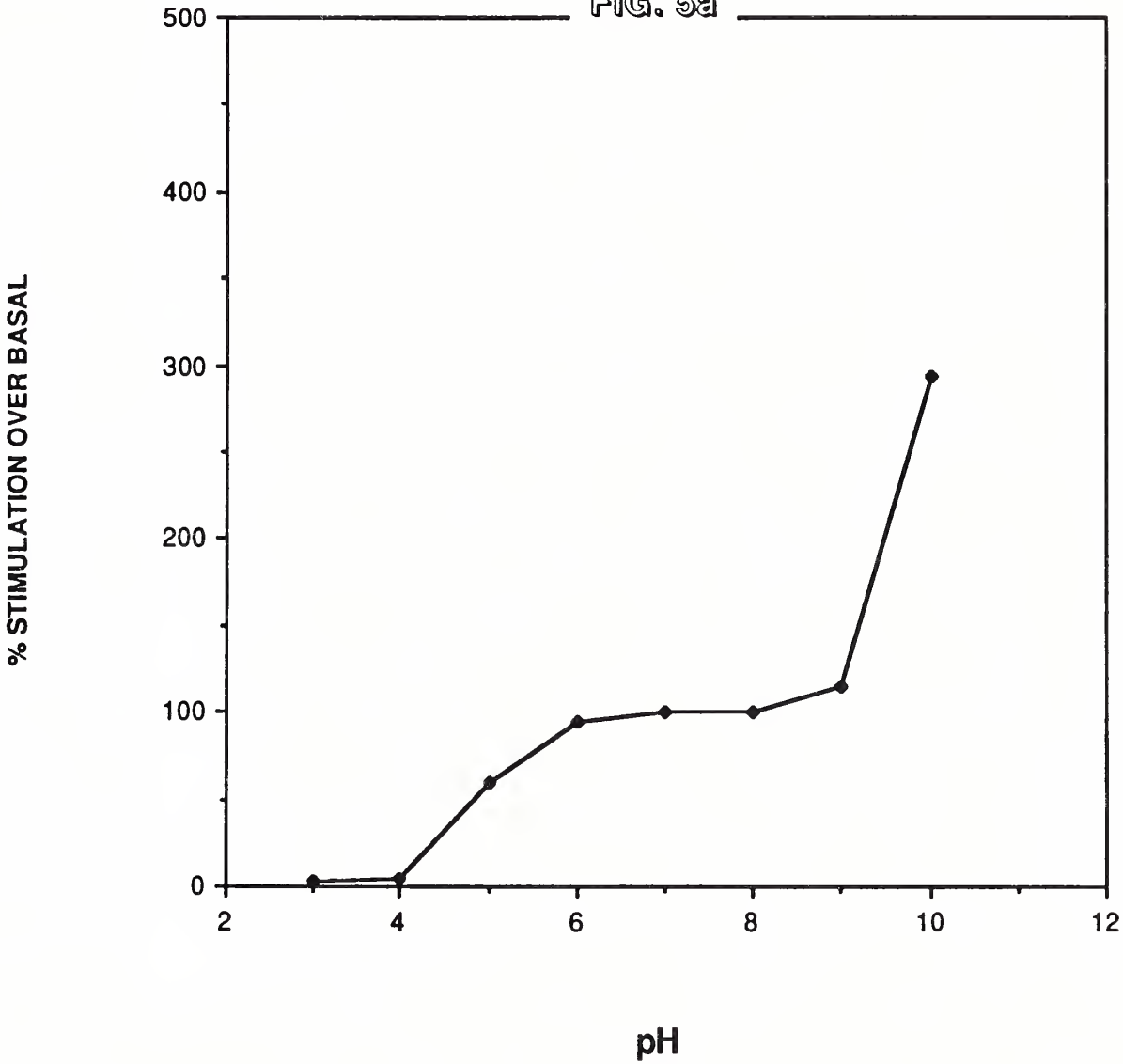


FIG. 5b

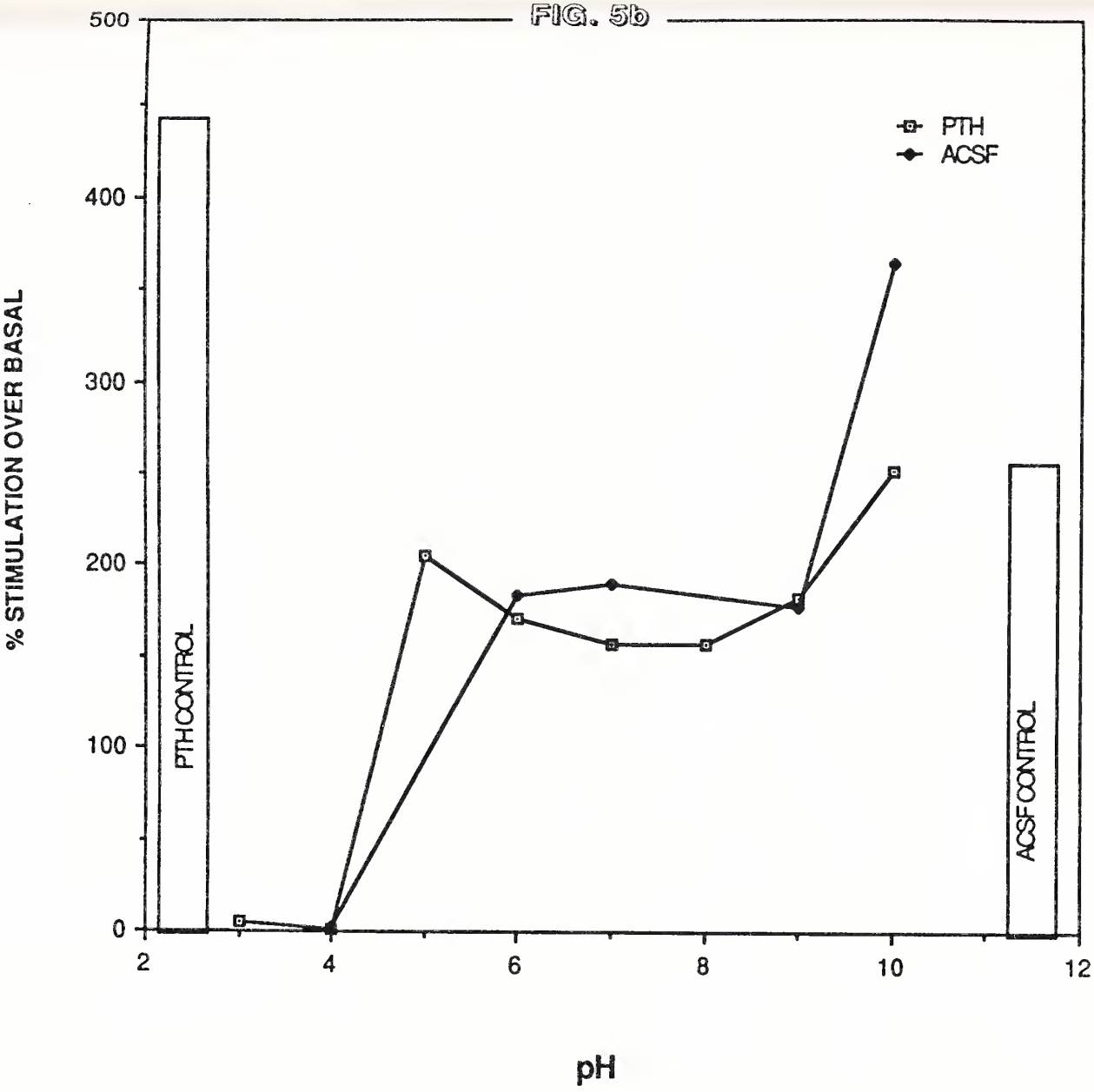

500
400
300
200
100
0
% STIMULATION OVER BASAL
PTH
ACSF
PTH CONTROL
ACSF CONTROL
2
4
6
8
10
12
pH

FIG. 5c

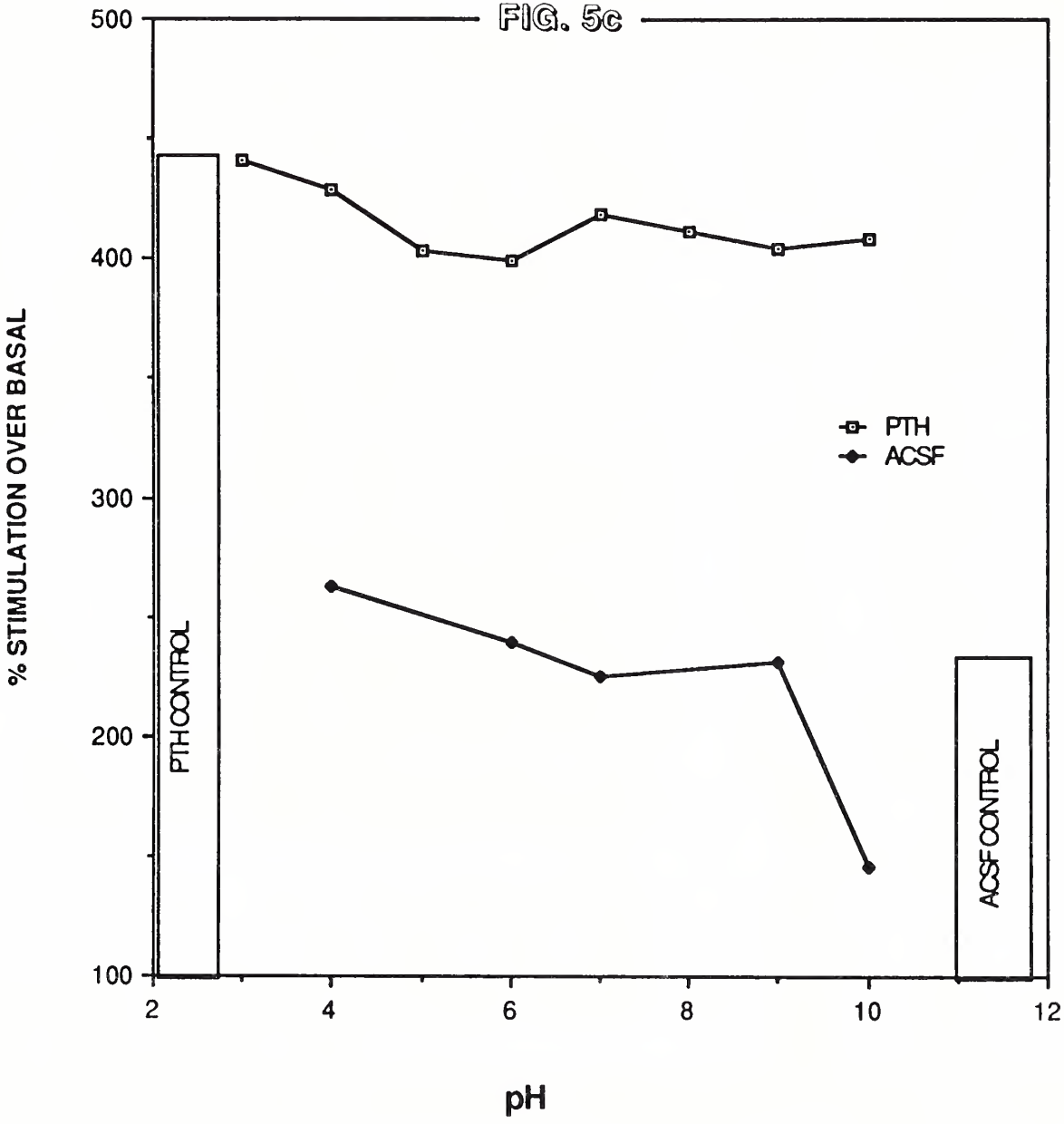

500
400
300
200
100
% STIMULATION OVER BASAL
PTH CONTROL
PTH
ACSF
ACSF CONTROL
2
4
6
8
10
12
pH

Figure 6

Results of Lectin experiments with ACSF.

- 6a. As described in Methods, ^{125}I radiolabelled *B* -HCG was used as a positive control for the Concanavalin A column. The figure represents a profile of cpm recovered from the various loading, washing, and eluting fractions applied to the column.

- 6b. This figure represents a profile of ACSF bioactivity recovered from the various loading, washing, and eluting fractions applied to the Concanavalin-A column.

- 6c. This figure displays the profile of ACSF bioactivity recovered from the loading sample buffer, the two washes, and the three elutions applied to the Wheat Germ Lectin column.

FIG 6a

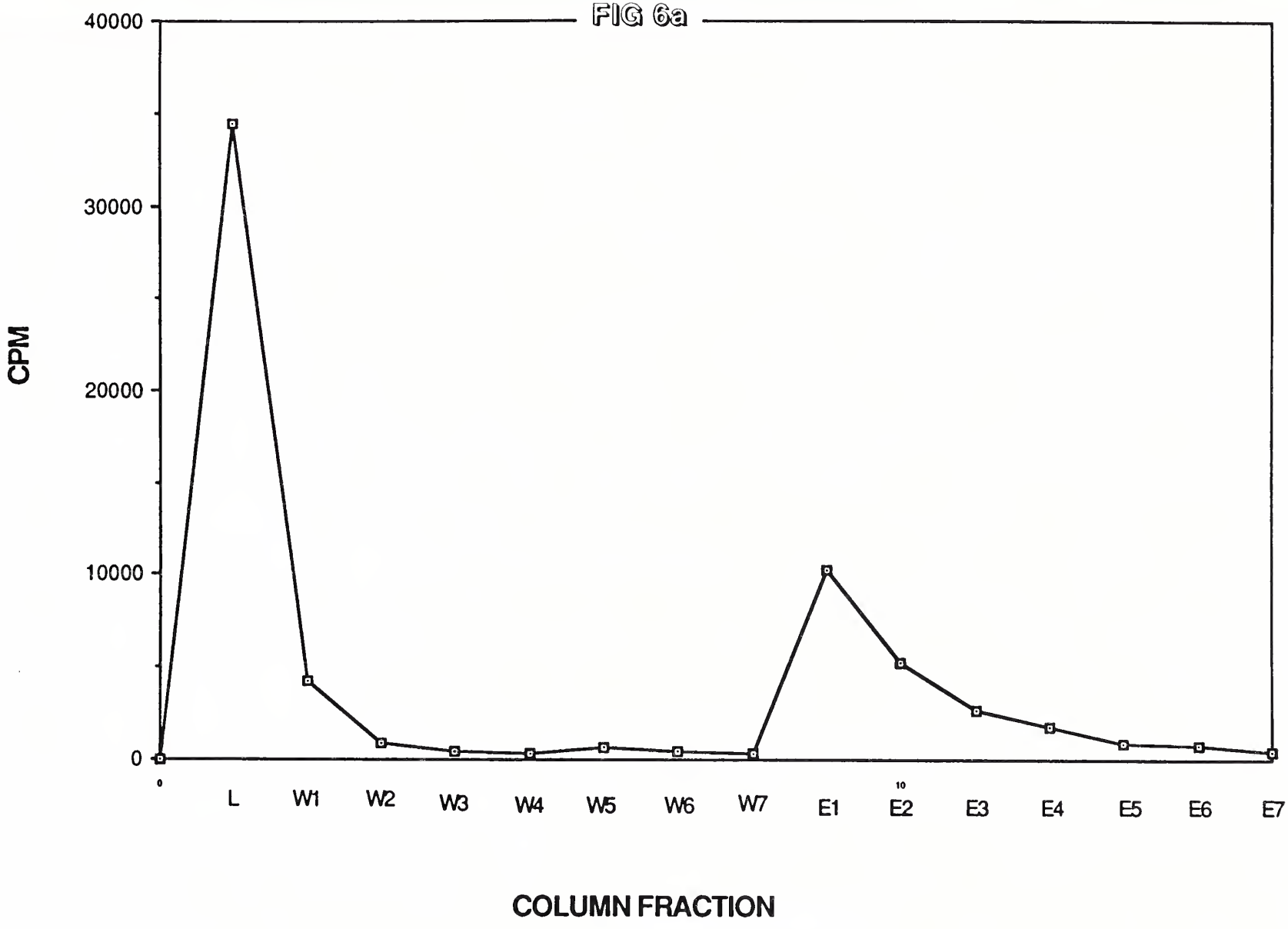

40000
30000
20000
10000
0
CPM
0
L
W1
W2
W3
W4
W5
W6
W7
E1
10
E2
E3
E4
E5
E6
E7
COLUMN FRACTION

FIGURE 6B

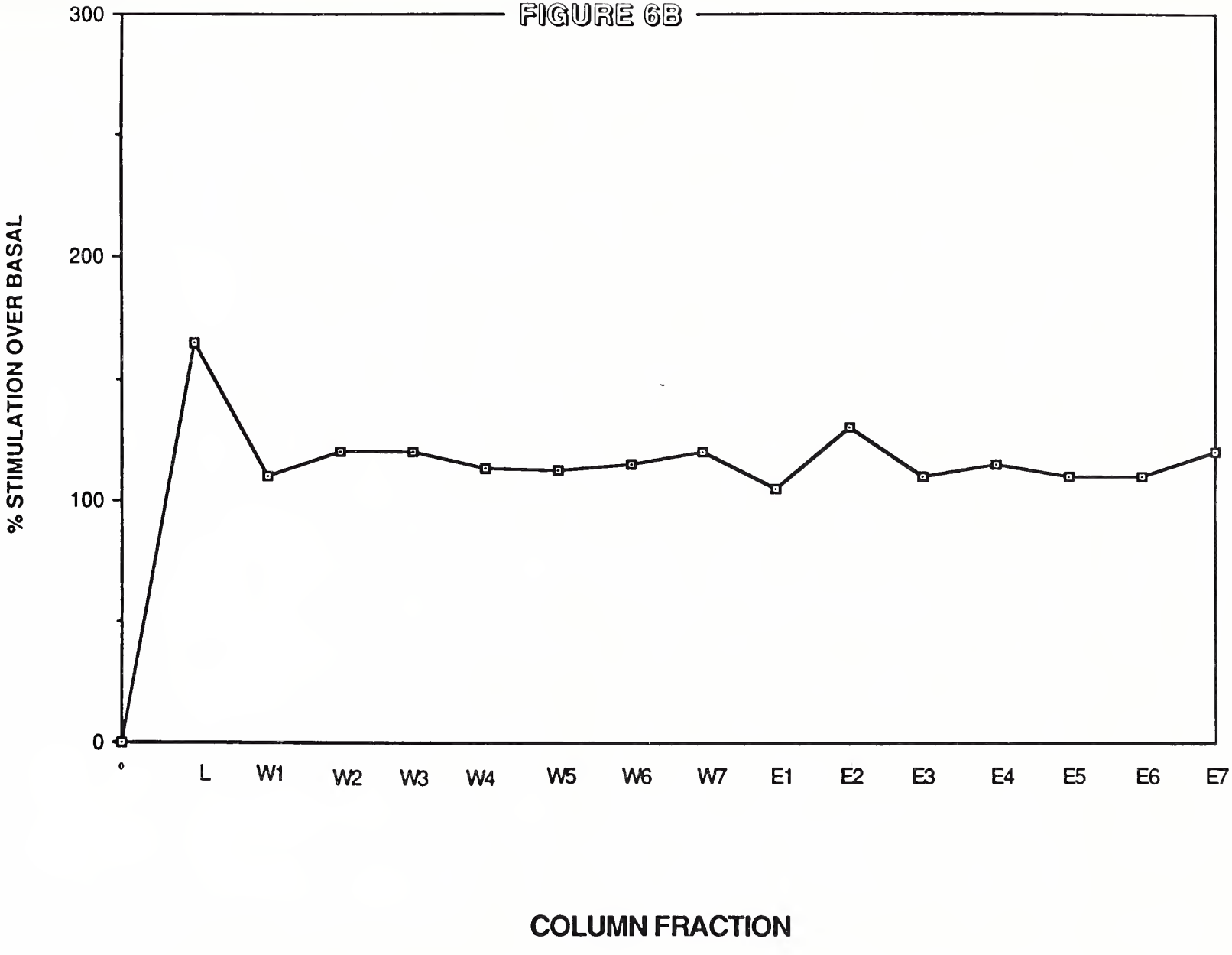
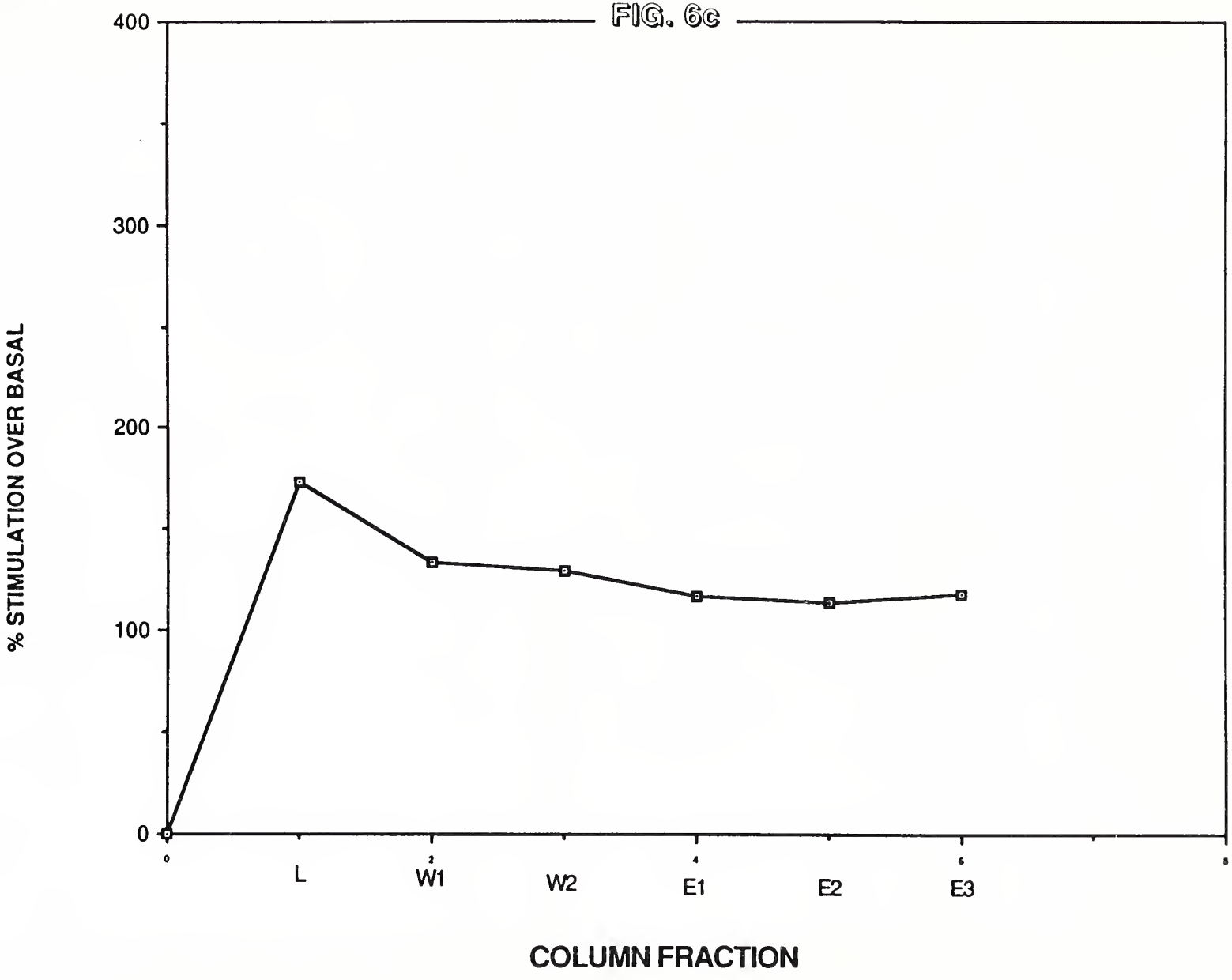

300
200
100
0
% STIMULATION OVER BASAL
°
L
W1
W2
W3
W4
W5
W6
W7
E1
E2
E3
E4
E5
E6
E7
COLUMN FRACTION

FIG. 6c


400
300
200
100
0
% STIMULATION OVER BASAL
L
W1
W2
E1
E2
E3
COLUMN FRACTION

DISCUSSION

Several laboratories are currently attempting to identify and to purify the factor or factors responsible for HHM. The experiments described herein were undertaken in pursuit of these goals. They were designed to investigate how the adenylate cyclase-stimulating factor associated with HHM behaves when it is exposed to a series of different physical and chemical conditions. The information gleaned from these experiments has been valuable in three respects. First, it has helped to describe more fully the physical characteristics of the factor(s). Second, it has provided some valuable guidelines in constructing purification schemes. Finally, it has allowed comparison of the adenylate cyclase stimulating activity and TGF activity, the two leading etiologic contenders proposed to be responsible for HHM.

The partially purified ACSF used in these studies causes potent dose-dependent stimulation of the renal cortical adenylate cyclase assay. As previously observed (120,125-128,137), the dose-response relationship is essentially identical to that given by PTH. Since the averaged dose-response curvies of ACSF and PTH so closely parallel each other, at least at the concentrations tested, the two proteins probably stimulate the adenylate cyclase system in an identical fashion. This fact lends further support to the notion that the ACSF interacts directly with the PTH receptor.

The ACSA associated with HHM is not sensitive to chemical reduction. L-cysteine and DTT exert an adverse effect on the renal adenylate cyclase assay. However, when these agents are diluted out so that they no longer affect the assay, none of the three reducing agents employed diminishes the bioactivity of

the ACSF. The concentrations of the three reducing agents employed, 300mM BME, 130 mM L-cysteine, or 65mM DTT, represent severe reducing conditions. Each of these conditions has been shown to be capable of dissociating disulfide-linked subunits of other purified and partially purified protein hormones (114,138,139). Therefore, it can be stated with some certainty that disulfide bonds are not crucial to the adenylate cyclase-stimulating activity associated with HHM.

Lack of sensitivity to reducing agents stands at odds with the descriptions of TGF activity reported by Mundy and his laboratory. TGFs in general are susceptible to reducing agents, and reports implicating TGFs as pathogenetic factors in HHM have stressed susceptability to the reducing agent DTT (114-118). This difference in behavior with respect to reducing conditions demonstrates that the TGF and adenylate cyclase bioactivities associated with HHM have different primary and tertiary structures. These bioactivities may reside in completely different peptides or they may reside in different regions of the same molecule. Research currently underway in Dr. Stewart's laboratory suggests that part of the adenylate cyclase stimulating protein not directly associated with the ACSA may contain disulfide bonds. Preliminary SDS-PAGE extraction studies indicate that under reducing conditions (50mM DTT), the adenylate cyclase-stimulating factor(s) may dissociate into subunits, one which contains the ACSA and one which does not (Stewart A, Burtis W, unpublished observations). It is interesting to speculate whether both bioactivities may reside in the same molecule, the TGF activity dependent upon disulfide bonds, the ACSA activity independent of such bonds.

In contrast to reduction, ACSA is sensitive to oxidation by hydrogen peroxide. Furthermore, this oxidation seems to be irreversible. PTH is also

sensitive to hydrogen peroxide as has previously been shown (139). However, PTH has been shown to regain its bioactivity with subsequent reduction (139). Under conditions of acidic pH, oxidation by hydrogen peroxide is fairly specifically targeted to methionine residues (140,141). Although the pH of these reactions was not controlled, the buffer containing the ACSF is a dilute acid (5mM acetic acid). Hence, the oxidation reaction most likely altered methionine residues. Other possible but less likely amino acid targets are cysteine, tyrosine and tryptophan (141). The observed susceptibility to hydrogen peroxide suggests that one of these four amino acids is important in maintaining the integrity of the ACSF. The most likely of the four is methionine, but methionine oxidation should be reversible upon subsequent reduction (141). It may be that there is some impediment to refolding of the protein resulting from the manner in which the reduction reactions were performed, or from features inherent in the primary structure of the molecule. An alternative is that oxidation causes an ireversible change in the protein, perhaps by altering one of the three amino acids other than methionine, mentioned above.

It is interesting to compare the time courses of oxidation for PTH and ACSF. ACSF loses its bioactivity very gradually over several hours. PTH is completely inactivated much more quickly, within thirty minutes. This difference might be explained in one of two ways. First, ACSF may be a more complexly folded protein with the oxidative targets partially protected within the interior of the molecule. On the other hand, it may be that PTH is affected by the rapid oxidation of methionine, while ACSF bioactivity is affected by the slower oxidation of some other amino acid. Whatever the molecular basis for the behavior of ACSF, it is clear that care should be taken to prevent oxidation during the purification and handling of the peptide.

In their attempts to further purify ACSF, Stewart and his associates have employed ion-exchange chromatography. However, using this method they experienced substantial loses of bioactivity. Preliminary experiments suggested that the ACSF might be unstable at the neutral pH's involved in these studies. For this reason, the activity of ACSF at different pH's was examined in detail. It is clear from these experiments that the buffer system used, 100 mM ammonium acetate, exerts both a pH-dependent inhibition and stimulation of the renal adneylate cyclase assay. There is profound suppression of cAMP formation at the most acidic values and there is autonomous stimulation of cAMP production at the most basic values. In light of these effects, it is difficult to interpret the experiments in which the sample was added to the assay dissolved in ammonium acetate. It is impossible to tell whether the observed loss of bioactivity is due to the ammonium acetate itself, or to a pH induced changed in the ACSF. If there is such a change in the ACSF, it is a reversible one. In the set of experiments in which the buffer is lyophilized before the sample is assayed, there is full recovery of bioactivity. The only exception is at pH 10, where there is loss of ACSF bioactivity. This loss of activity may be due to partial denaturation of the protein (142) or perhaps to a conformational change secondary to the loss of charge on a basic amino acid(s). In any case, it seems unlikely that the poor recovery from ion exchange chromatography can be explained by any pH-dependent, irreversible changes in the protein. The losses more likely were due to irreversible binding of ACSF to the ion-exchange column.

ACSF was digested with cyanogen bromide in the hopes of producing a peptide fragment that retained biological activity. Such a fragment would be more easily sequenced, and the sequence obtained would be of the biologically active part of the molecule. The digests did retain some activity in the

adenylate cyclase assay, but HPLC analysis demonstrated that the activity represented residual undigested ACSF. It is interesting that under the conditions used in the experiments, PTH was completely digested while ACSF was not. This mirrors the results of the oxidation experiments. Since CNBr cleaves peptide chains at methionine residues (143), these experiments again suggest that this amino acid may be important to the protein's bioactivity and that it might be located in a semi-protected domain in an interior portion of the molecule. These CNBr experiments are but initial steps in the effort to generate an active fragment. It will be important to try more gentle CNBr conditions as well as site-specific enzymatic reactions in an attempt to partially digest the protein.

Glycoproteins are ubiquitous in nature. Therefore, it is reasonable to inquire whether the ACSF might be such a molecule. However, since the factor is not yet purified to homogeneity, submitting it to the available chemical methods for the detection of carbohydrate content would be of limited value. If carbohydrate were detected, it would not necessarily have been liberated from the adenylate cyclase-stimulating protein. Another way to approach this problem is to attempt to specifically bind and elute the adenylate cyclase-stimulating bioactivity to and from a lectin carbohydrate affinity column. Lectins are proteins of nonimmune origin found in plants and microorganisms that have the ability to noncovalently bind certain carbohydrate moieties (144). Binding ACSF to such a column would both characterize it as a glycoprotein and also offer a quick and simple purification step. With this goal in mind, partially purified ACSF was applied to concanavalin A and wheat germ lectin, both covlently attached to Sepharose beads. Conconvalin A recognizes *a* -mannose and *a* -glucose residues; wheat germ lectin recognizes ß-N-acetyl glucosamine and *a* -N-acetylneuraminic acid (144). As shown in the results section, it did not seem

as if either of these lectins specifically bound the factor, suggesting that ACSF does not contain any of the 4 sugar residues mentioned above in accessible locations. However, the low recovery of activity from these experiments makes any interpretation perilous. Additional controls must be run to investigate the possibilities that the ACSA bound nonspecifically to the Sepharose beads or that the columns somehow destroyed the activity (perhaps via protease contamination). These experiments represent preliminary attempts and will be expanded to include different lectins with different carbohydrate binding specificities as well as the aforementioned controls.

With the experiments discussed above, the groundwork has been set to complete the final purification of the adenylate cyclase-stimulating factor or factors involved in HHM. This will allow a direct comparison of this protein to the TGF activity also implicated in the syndrome. With the recent finding of Burtis and Stewart, that the protein appears to be composed of subunits, one having ACSA and the other not having this activity, it is entirely possible that both the TGF activity and the ACSA activities reside in the same molecule. This hypothesis might also explain the disparity in the way in which the two bioactivities respond to reducing conditions. Perhaps the TGF activity requires the integrity of the disulfide bonds linking the two subunits together; the adenylate cyclase stimulating activity apparently does not. Whether or not the ACSF is eventually shown to possess TGF activity, it seems likely that its significance will go beyond the realm of HHM. The work of Merendino et. al. (132) suggests that it plays a role in physiology as well as pathophysiology. The question of its exact role in skin physiology warrants further investigation, a task which will be much more easily accomplished once the protein is purified and sequenced.

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