IMMUNOCHEMICAL IDENTIFICATION OF PARATHYROID HORMONE IN NON-PARATHYROID NEOPLASMS ASSOCIATED WITH HYPERCALCEMIA*, ‡

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(Received for publication, November 25, 1963)

The association of the clinical and laboratory indications of primary hyperparathyroidism with malignant non-parathyroid tumors not metastatic to bone has been reported in at least 18 cases (1, 2). The rapid disappearance of hypercalcemia following removal of the tumor and the reappearance of an elevated serum calcium on recurrence of the tumor have been considered indirect or circumstantial evidence that the neoplasms themselves may produce a parathyroid hormone-like substance (1, 2). Demonstration of ectopic ACTH in non-pituitary neoplasms associated with Cushing's syndrome, and the finding of other hormone-like substances in non-endocrine tumors (3, 4) have established the concept that a variety of neoplastic tissues may display endocrine activity.

Previous attempts to use biological assay methods to identify directly a parathyroid hormone-like substance in malignant tumors were unsuccessful probably because of the insensitivity of the methods used. The availability of an antiserum to bovine parathyroid hormone (5), and the finding of immunochemical cross-reactivity between extracts of bovine and human parathyroid glands with anti-bovine antibody (6) have enabled us to detect, by quantitative complement (C′) fixation, an antigen reacting with antiparathyroid hormone in 6 tumors obtained from patients with the hypercalcemic syndrome. This C′ fixation assay method is several hundred times more delicate than the biological assay methods previously used to assay such neoplasms.

The immunochemical evidence that the tumor antigen is either human parathyroid hormone or a very closely related molecule is presented in this report.

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* Contribution No. 266 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts.

‡ This investigation was supported in part by research grants from the National Institute of Arthritis and Metabolic Diseases (A-1787), and the American Cancer Society(E-222).

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In addition to offering the first direct evidence for production of parathyroid hormone by non-parathyroid neoplasms, these data also demonstrate serologic differences between human and bovine parathyroid hormones.

Materials and Methods

Parathyroid Hormone Preparations.—Two highly purified parathyroid hormone preparations were used. They were prepared from phenol extracts of acetone powder of bovine parathyroid glands (Wilson Labs., Chicago), fractionated by the method of Aurbach (7) to the stage of the trichloroacetic acid precipitate.

Preparation A, used as the immunizing antigen, was further purified by two successive gel filtrations through columns of sephadex G-50, following the procedure of Rasmussen and Craig (8). Amino acid analyses[1] by the method of Spackman *et al.* (9) were in good agreement with those previously reported by Rasmussen and Craig (8) for highly purified bovine parathyroid hormone. The specific activity of preparation A in the bioassay method of Munson (10) was as high as or higher than any previously reported, 40,000 $\overset{\times}{\div}$ 1.21 units/mg nitrogen.[2] Protein nitrogen was estimated by the method of Lowry *et al.* (11).

Preparation B was purified by an independent method, countercurrent distribution, using the salt system of Aurbach (7).

Antiserum.—One mg of parathyroid hormone preparation A, dissolved in 1.0 ml of 0.005 N acetic acid and emulsified with 1.0 ml of complete Freund's adjuvant was administered to a single albino rabbit by simultaneous toe-pad and intramuscular injections. Beginning 3 weeks after the initial immunization, an intravenous injection of 150 μg of the same antigen was given daily for 5 days. The rabbit was bled 1 week after the last intravenous injection, and the serum recovered was stored at −20°C without preservative.

Quantitative Complement (C′) Fixation.—Quantitative C′ fixation was performed as previously described (5). In the present experiments the diluent was isotonic NaCl-tris-(hydroxymethyl)-aminomethane buffer, pH 7.3, containing 5×10^{-4} M $MgCl_2$ and 1.5×10^{-4} M $CaCl_2$ but no added protein. The dilution of antibody used is given in the description of each specific experiment. C′ fixation inhibition was performed as described by Wasserman and Levine (12). Before addition to the final reaction mixture the diluted antigens were subjected to sonic oscillation for 3 minutes (5).

Immunochemical Homogeneity.—On vertical starch gel electrophoresis of the immunizing antigen (preparation A) a dense, narrow protein band appeared with the characteristic mobility of parathyroid hormone as shown previously by immunological and biological assays of gel eluates (5). A second very weak band corresponded to material that was biologically and immunologically inactive. Hormone preparation B, on electrophoresis in the same starch gel system, migrated as a single, dense, narrow band with the same mobility as the major biologically and immunologically active band obtained with the gel filtration product (preparation A) (5). Identical C′ fixation curves were obtained with the two preparations. Double diffusion in agar, for 72 hours at 4° or 20°C, of the immunizing preparation, crude parathyroid gland extracts (300 units/mg N), and undiluted or even 4 times concentrated antiserum, gave only a single band of precipitation (5). Complement fixation in far antigen excess gave no evidence of a second immune system (5). The good agreement between immunoassays (using this antiserum) and bioassays of parathyroid hormone preparations of widely varying

[1] The empirical formula was: lys_7, his_3, arg_4, asp_7, thr_1, ser_6, glu_9, pro_3, gly_4, ala_7, val_5, met_1, $ileu_2$, leu_8, tyr_1, phe_2, try_1. We are greatly indebted to Dr. Helen Van Vunakis and Mr. Fred Castillo, Graduate Department of Biochemistry, Brandeis University, for these analyses.

[2] The factor following the symbol $\overset{\times}{\div}$, when multiplied by and divided into the potency estimate, gives the limits of the standard error.

potencies (5) is further evidence of the homogeneity and specificity of the antiserum. The use of rabbit antiserum against parathyroid hormone to precipitate the highly purified hormone from solution and the recovery of the biological activity from the immune precipitate, have been reported previously (13).

Biological Assay.—In this paper the term biological activity refers to activity in the assay method of Munson (10), which is based on the maintenance of the serum calcium level in acutely parathyroidectomized rats. The results were evaluated by standard statistical procedures for parallel-line assays according to Finney (14).

TABLE I

Clinical Data

Case No.	Tumor		Parathyroid glands	Serum			Other
	Type	Metastasis to		Calcium	Inorganic phosphate	Alkaline phosphatase	
				mg/100 ml		*units*	
1	Renal cell		Not examined	14.0–15.4	2.9–4.5	4.1–6.2 (Bodansky)	
2	Renal cell*	Lung	Atrophic	11.4–18.6	1.8–3.6	0.9–3.7 (Bessey-Lowry)	TRP‡ = 68 per cent
3	Pancreas	Liver	Normal	17.0	—	—	
4	Renal cell		Normal	>14.0	<3.0	—	
5	Lung		Not examined	9.6–15.4	1.2–3.0	1.7–3.5 (Bessey-Lowry)	TRP = 73 per cent
6	Colon§	Liver	Normal	15.2–18.6	2.0	16.5 (Bodansky)	

* For a detailed description of the clinical aspects of this case see Goldberg *et al.* (2).

‡ Tubular reabsorption of phosphate.

§ See Case Records of the Massachusetts General Hospital: Case 63-1963, *New England J. Med.*, 1963, **269**, 801.

Tissue Extracts.—

(*a*) *Urea extracts of human and bovine parathyroid tissue:* Normal human parathyroid glands obtained at autopsy or human parathyroid adenomas were homogenized with acetone (500 ml/gm) and then stirred for 24 hours at 4°C. The powder was obtained following filtration and washing with fresh acetone. Either commercial acetone powders or freshly ground whole parathyroid glands were the source of bovine material. The powders or ground tissue were stirred with aqueous 8 M urea (20 ml/gm) for 2 to 3 hours at 25°C. The insoluble residue was removed by centrifugation at 3000 *g* for 15 minutes at 4°C and the clear supernatant was stored at 2–4°C. Storage in the frozen state was avoided because an insoluble precipitate was found after thawing and the immunological activity had decreased.

(*b*) *Dilute hydrochloric acid extracts of bovine and human parathyroid tissue:* Normal bovine and human parathyroid glands and human parathyroid adenomas, freshly ground or acetone powders, were stirred with 0.1 N HCl (10 ml/gm) for 15 minutes in a water bath at 100°C. Lipid was removed from the extracts by filtration through glass wool and the insoluble ma-

terial, separated by centrifugation at 3000 *g* for 15 minutes at 4°C, was discarded. Some of the preparations were adjusted to pH 11.0 with 5 N NaOH and then back titrated to pH 8.0 with 1 N HCl. Any insoluble material was again removed by centrifugation. This simple purification step, which did not alter the serological activity, facilitated testing for C′ fixation at relatively low dilutions. These extracts were stored at −20°C.

(*c*) *Phenol extracts of human parathyroid tissue:* Acetone powders of human parathyroid tissue (prepared as described above) were stirred with 88 per cent phenol (10 ml/gm) containing 0.05 M 2-mercaptoethanol. The mixture was then carried through the next step in the Aurbach method for preparation of bovine parathyroid hormone (7), the formation of the ether precipitate; the precipitate was then taken up in 0.01 N HCl. Any material not in solution after 3 hours' stirring at 4°C was removed by centrifugation and the clear supernate was stored at −20°C.

All extracts of both human and bovine parathyroid glands tested in this study were biologically active.

(*d*) *Extracts of human neoplasms and control tissues:* Tumor and control (non-tumor) tissues were obtained at surgery or autopsy from 6 hypercalcemic patients who had no evidence (clinical, radiological, or pathological) of osseous metastases. Table I shows the pathological and clinical laboratory data from these patients. In addition, 7 human tumors,—carcinomas of pancreas (two), lung, colon (two), breast, and a neuroblastoma,—obtained from patients without hypercalcemia were obtained and treated in an identical manner. The specimens were stored at −20°C until extraction. Following partial defatting and dehydration with acetone (100 ml/gm, 24 hours, 4°C) 3 types of extracts were prepared from the resulting powders. Each specimen was extracted with aqueous 8 M urea exactly as described for parathyroid tissue. Phenol and dilute HCl extracts of certain specimens were likewise prepared in a manner identical with that described for parathyroid glands. Urea extracts were stored at 4°C until assay (less than 48 hours later). The phenol and HCl preparations were kept frozen until use.

RESULTS

Serologic Activity of Bovine Parathyroid Hormone Obtained by Three Different Extraction Procedures.—Figs. 1 *A* and 1 *B* show, respectively, the C′ fixation obtained with the highly purified phenol-extracted bovine parathyroid hormone and with an 8 M urea extract of bovine parathyroid tissue at an antibody dilution of 1/1500. The HCl extract lacked the capacity to fix C′ with this dilution of antiserum, but it did react with the antiparathyroid hormone as measured by inhibition of C′ fixation by the homologous (phenol-extracted) parathyroid hormone–antiparathyroid hormone (Fig. 1 *C*).

Serologic Activity of Human Parathyroid Gland Extracts Obtained by Three Different Extraction Procedures.—Direct C′ fixation between the urea extract of human parathyroid glands and antibovine hormone was seen at an antibody dilution of 1/100 (Fig. 2 *A*). No C′ fixation was observed with the antiserum dilution used to detect homologous antigen (1/1500); however, this urea extract did inhibit the homologous C′ fixation at an antiserum dilution of 1/1500. As in the bovine system, the HCl extract of human glands did not fix C′ directly even at an antibody dilution of 1/100 (Fig. 2 *A*). It was, however, a potent inhibitor of the bovine system (Fig. 2 *B*). Phenol extracts of human parathyroid tissue also did not fix C′ directly, but did show 100 per cent in-

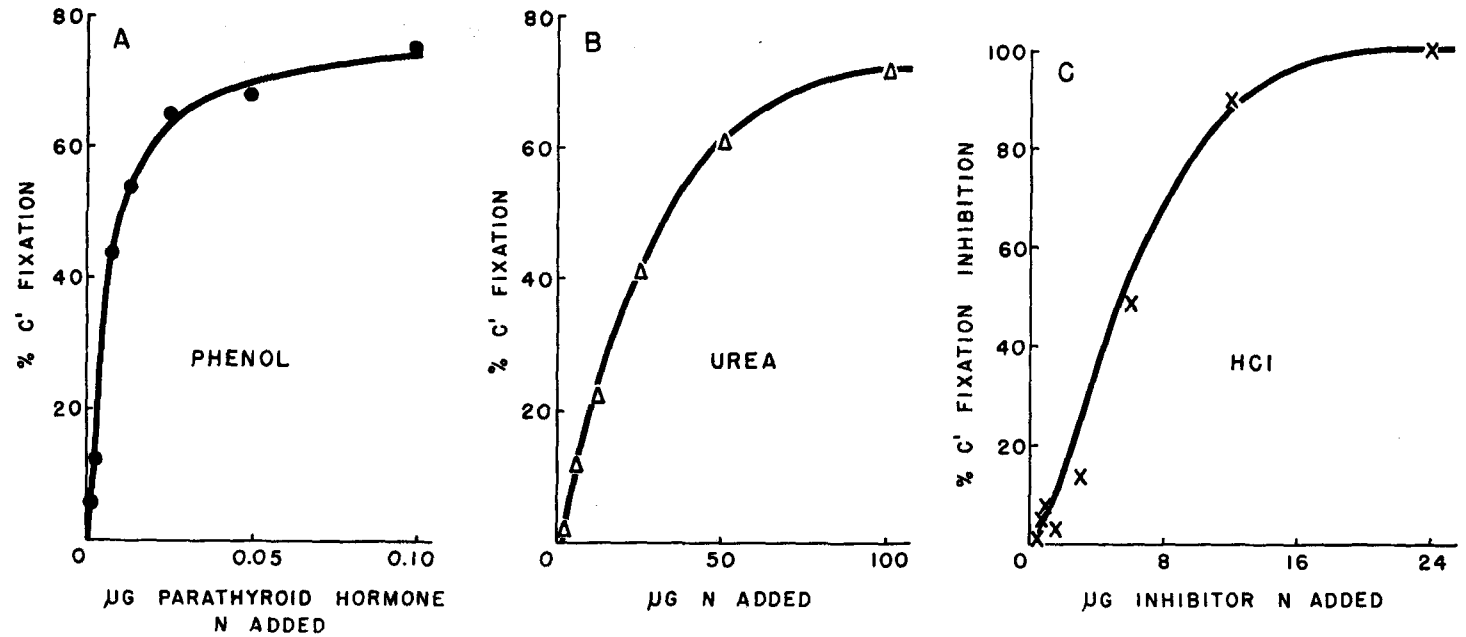

FIG. 1. Complement fixation and C′ fixation inhibition between extracts of bovine parathyroid tissue and anti-bovine parathyroid hormone, diluted 1/1500. *A*, phenol-extracted, highly purified, parathyroid hormone. *B*, 8 M urea parathyroid gland extract. *C*, C′ fixation inhibition with a 0.1 N HCl bovine gland extract. In this experiment as well as in the subsequent inhibition experiments (with the exception of that shown in Fig. 3) the C′ fixation being inhibited was that obtained with 0.06 μg N of pure bovine parathyroid hormone. The HCl extract gave no direct C′ fixation at this 1/1500 dilution of antiserum.

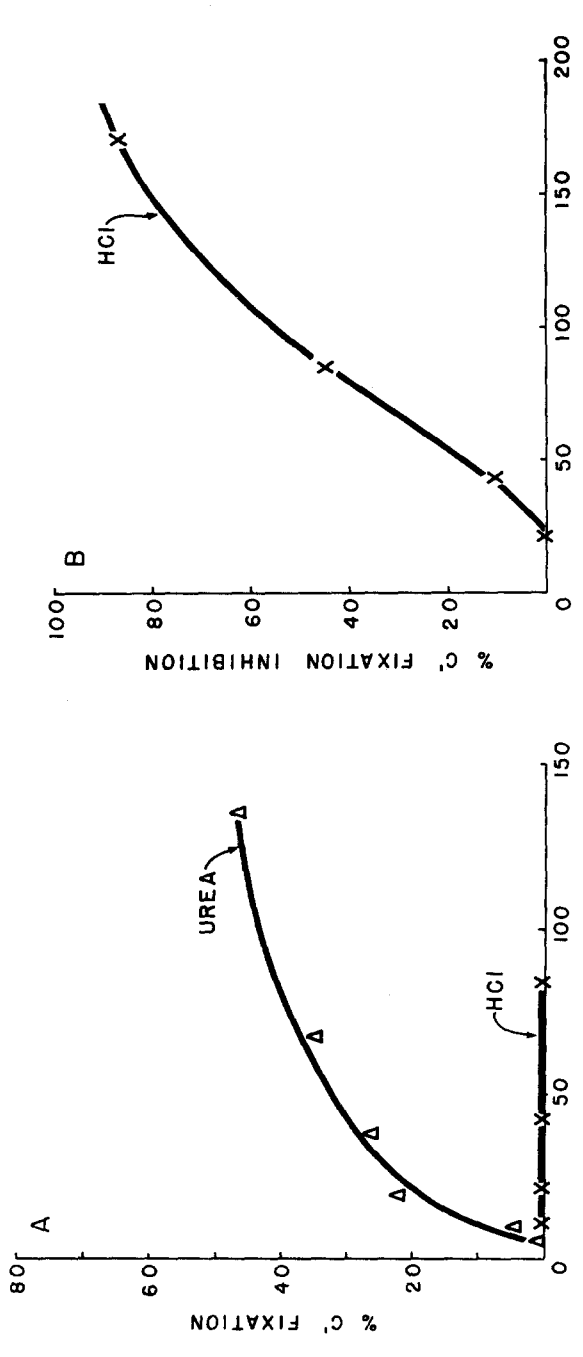

FIG. 2. Complement fixation and C′ fixation inhibition between extracts of human parathyroid tissue and anti-bovine parathyroid hormone. *A*, 8 M urea and 0.1 N HCl extracts, antiserum diluted 1/100. *B*, C′ fixation inhibition with the 0.1 N HCl human gland extract, antiserum diluted 1/1500. Phenol extracts of human parathyroid tissue, like the HCl extract shown, did not fix C′ directly, but they were potent (up to 100 per cent) inhibitors of the homologous bovine system even at an antiserum dilution of 1/400.

hibition (at an inhibitor concentration of 10 μg N) of the homologous system even at an antibody dilution of 1/400. It is possible that the lack of direct C′ fixation with phenol extracts of human glands, as well as the need for a higher concentration of antibody for direct reaction with urea-extracted human antigen than for bovine hormone, reflect alterations in the human hormone during extraction. However, because of the relatively mild conditions of these extraction methods applied similarly to both species the more reasonable explanation is that the antigenic sites on the human molecule, although similar to, are not identical with those sites on the bovine hormone, thus giving rise to true immunochemical cross-reactivity frequently observed between organ-specific proteins of different species (15). Unpublished data obtained in our laboratory with phenol and HCl extracts of *rat* parathyroid glands also show immunochemical cross-reactivity, but not identity, with *bovine* hormone. Neither type of rat extract fixed C′ directly with anti-bovine antibody, although again each of them was a potent inhibitor of the homologous system.

Serologic Activity of Human Tissue Extracts.—Fig. 3 *A* shows the direct C′ fixation obtained with urea extracts of the tumors obtained from six hypercalcemic patients. The antibody dilution was 1/200. Also shown is the lack of direct C′ fixation with the urea extract from case 1 at an antiserum dilution of 1/1000 (Fig. 3 *A*). The finding that the human parathyroid gland extract (urea) fixed C′ at an antibody dilution of 1/100 while the tumor extracts reacted at 1/200, does not necessarily demonstrate lack of identity between these two antigens. It is more likely that this difference reflects the small concentration of antigen in the parathyroid tissue examined, placing this reaction far into the zone of antibody excess. The shape and rising nature of the C′ fixation curve for the human gland extract (Fig. 2 *A*) are fully compatible with this reasoning. At high concentrations the anticomplementary properties of the tumor extracts prevented attainment of peak fixation (cases 3 to 6) and description of the zone of antigen excess inhibition (all cases). In the three cases (Nos. 2, 3, and 6) in which metastatic tumor was available for assay, urea extracts reacted directly with antibody (Fig. 3 *A* and Table I) while extracts of the tissue immediately adjacent to the metastasis gave *no* C′ fixation. Likewise, urea extracts of uninvolved organs from the same cancer patients gave uniformly negative reactions. The antigen was also not detected in any of the urea extracts of the 7 neoplasms not associated with hypercalcemia.

As seen in Fig. 3 *B*, urea extracts of tumor also inhibited the homologous reaction even at an antibody dilution of 1/1000. Control extracts were ineffective as inhibitors. Both HCl and phenol extracts of tumors, like these two types of extracts of human parathyroid glands, failed to fix C′ directly at an antiserum dilution of 1/200, but they did retain the capacity to inhibit the bovine hormone-antihormone system. Fig. 4 shows this inhibition by an HCl extract at two antibody dilutions.

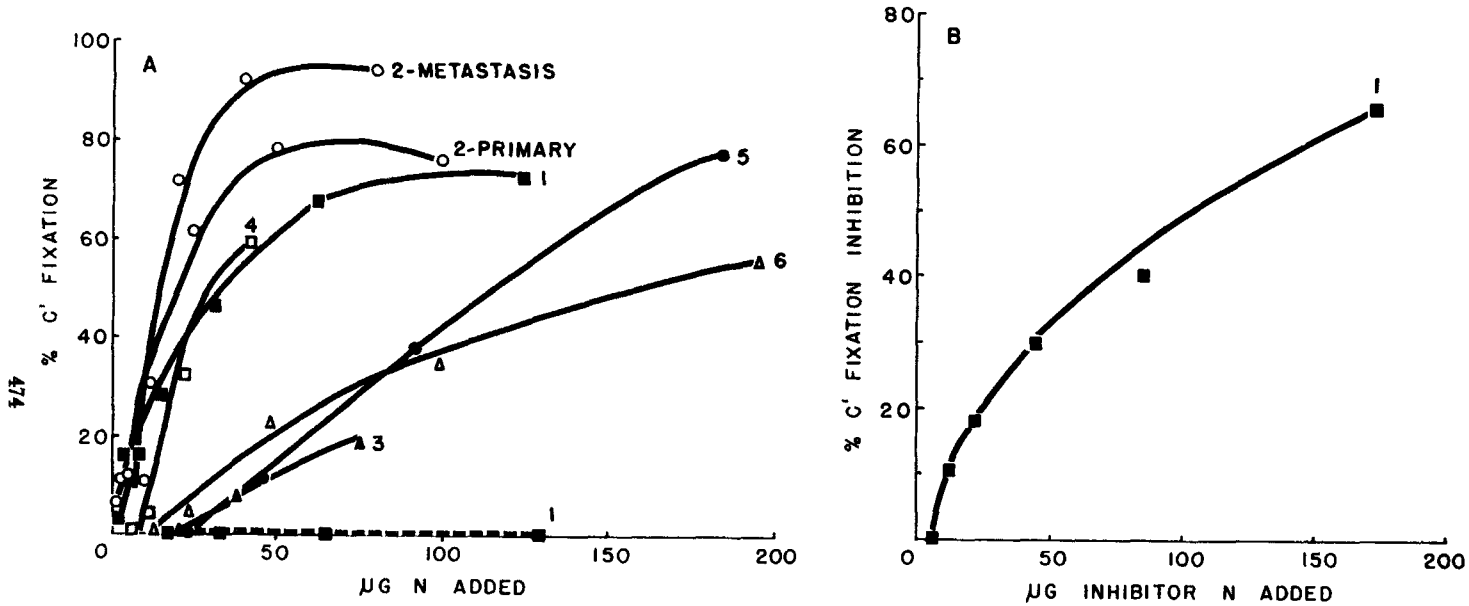

FIG. 3. Complement fixation and C′ fixation inhibition between urea extracts of non-parathyroid human neoplasms from hypercalcemic patients and anti-bovine parathyroid hormone. *A*, direct C′ fixation with 8 M urea extracts of tumors and antiparathyroid hormone diluted 1/200 (solid lines), and with the extract from case 1 and antiparathyroid hormone diluted 1/100 (dashed line). The numbers 1 to 6, identifying the curves, correspond to the case No.'s listed in Tables I and III. The C′ fixation curves with extracts of both the primary and metastatic (to lung) tumor in case 2 are shown. *B*, C′ fixation inhibition with the urea extract (No. 1), antiserum was diluted 1/1000 and the concentration of the homologous antigen being inhibited was 2.5 µg N.

Tests for non-specific inhibition of C′ fixation by all types of tumor and parathyroid gland extracts with three other independent immune systems (egg albumen, pepsinogen, and hemoglobin) were entirely negative.

Absorption of Antiparathyroid Hormone with Tumor Antigen.—

In three experiments rabbit antiserum was absorbed with tumor extracts and tested for antibody titer against the homologous bovine antigen. In these experiments urea tumor ex-

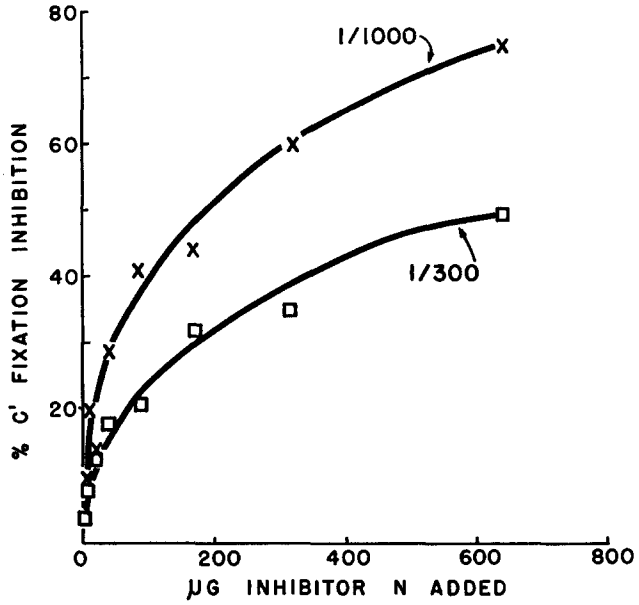

FIG. 4. Complement fixation inhibition by 0.1 N HCl extract of tumor (No. 4), antiserum diluted 1/300 and 1/1000. There was no direct C′ fixation with this HCl extract at either dilution of antiserum. Phenol extracts of the tumors, like the HCl extract shown, did not fix C′ directly, but they were potent (up to 100 per cent) inhibitors of the homologous bovine system at an antiserum dilution of 1/1500.

tracts were dialyzed in 8/32 Visking tubing twice against 400 volumes of 0.15 M NaCl at 4°C for 30 minutes each. An absorption mixture was then prepared in buffer which contained antiserum at a final dilution of 1/20, and an amount of tumor antigen calculated from the C′ fixation data (Fig. 3 *A*) to approximate the equivalence zone. Mixtures containing control tumors (not giving direct C′ fixation) plus antiserum, and control antiserum alone were handled identically. Following storage at 2°C for 2, 4, or 6 days, the reaction mixtures were centrifuged at 2000 RPM at 4°C for 60 minutes and the supernatant solutions were then diluted appropriately immediately before assay.

Figs. 5 *A* to 5 *C* show the results of these absorption experiments. A consistent decrease in titer (40, 49, and 40 per cent at peak C′ fixation) toward the homologous test antigen was observed in each experiment (Figs. 5 *A* to

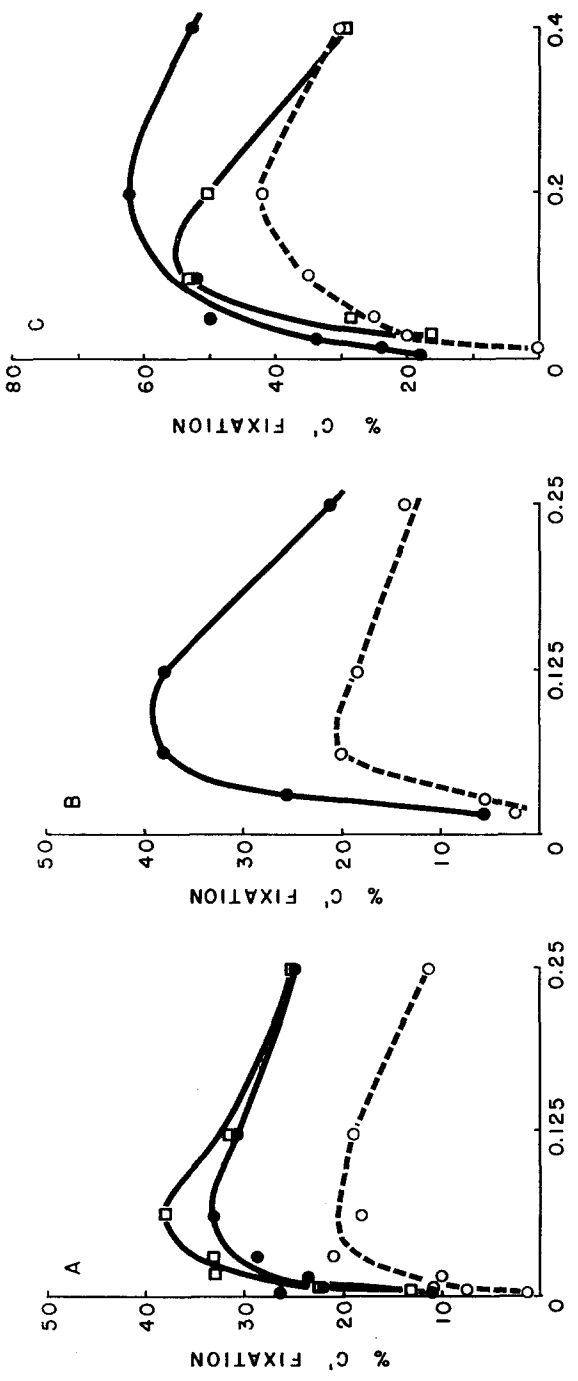

FIG. 5. Antiparathyroid hormone absorption. Three independent experiments showing C′ fixation between the homologous highly purified bovine parathyroid hormone and the antiparathyroid hormone after absorption with a tumor which gave direct C′ fixation (No. 1) (○——○), with a control tumor (●——●), and without absorption (□——□). *A*, incubation for 4 days, final antiserum dilution 1/1800. *B*, incubation for 6 days, final antiserum dilution 1/1500. *C*, incubation for 2 days, final antiserum dilution, 1/1600. An unabsorbed antiserum control was not included in *B*.

5 *C*). No loss in titer was observed in absorption with control tumors. No detectable antibody toward the tumor antigen remained in the supernatant solution after absorption at an antiserum dilution of 1/200.

To provide evidence that the antibody absorption experiments signify the removal of a cross-reacting rather than a contaminating antibody, it was necessary to show that C′ fixation, at a dilution of antiserum where a major system was being measured, was not quantitatively influenced by the presence of a minor independent antigen-antibody system not fixing C′ directly at that antibody concentration. This hypothesis was tested by preparing an artificial heterogeneous mixture of antibodies in which the proportion of minor to major antibodies was 1 to 5 as judged by direct C′ fixation. This ratio (1 to 5, or 20 per cent) was specifically chosen to exceed slightly the proportion of a hypothetical contaminating antibody in the parathyroid hormone system. If it were argued that the antibody reacting with the human tumor antigen at a dilution of 1/200 was a minor antibody, and that the antibody reacting with purified parathyroid hormone at a dilution of 1/1500 was a major, but independent, antibody, the ratio of these two systems would be 1 to 7.5 (13 per cent). Figs. 6 *A* and 6 *B* indicate, respectively, the homologous C′ fixation curves obtained with swine gastric pepsinogen and its antibody at a dilution of 1/4000 and chicken hemoglobin and its antibody at a dilution of 1/2000. No C′ fixation with hemoglobin was seen at an antibody dilution of 1/10,000 (Fig. 6 *C*). In the experiment shown in Fig. 6 *D*, the antigen was an artificial mixture of both pepsinogen and hemoglobin and the antibody was also a mixture of both antipepsinogen (1/4000) and antihemoglobin (1/10,000). The C′ fixation curve obtained (Fig. 6 *D*) was identical with the pepsinogen curve obtained in the absence of the second contaminating system (Fig. 6 *A*). Thus, the consistent decrease in C′ fixation in the parathyroid hormone system after absorption must represent removal of cross-reacting antibody.

Biological Assay.—The immunochemically active urea extracts of nonparathyroid tumors were tested in a preliminary manner for biological activity by the Munson method (10). As shown in Table II, there was a small but statistically significant effect consistent with the presence of parathyroid hormone. In order to increase the dose per rat in the bioassay the activity in the urea extracts was concentrated by precipitation with trichloroacetic acid (final concentration, 6 per cent). The precipitate was washed with ether and dissolved in a small volume of 0.1 N HCl.

Estimation of Parathyroid Hormone in Tumors.—Although the tumor antigen is related to the homologous bovine antigen it is not identical with it. Consequently, estimates of the antigen content of the tumors, using anti-bovine parathyroid hormone, can be expressed only in relative, not absolute, terms. The correction factor for conversion of these relative values to absolute values cannot be determined until the human tumor hormone is available in a homo-

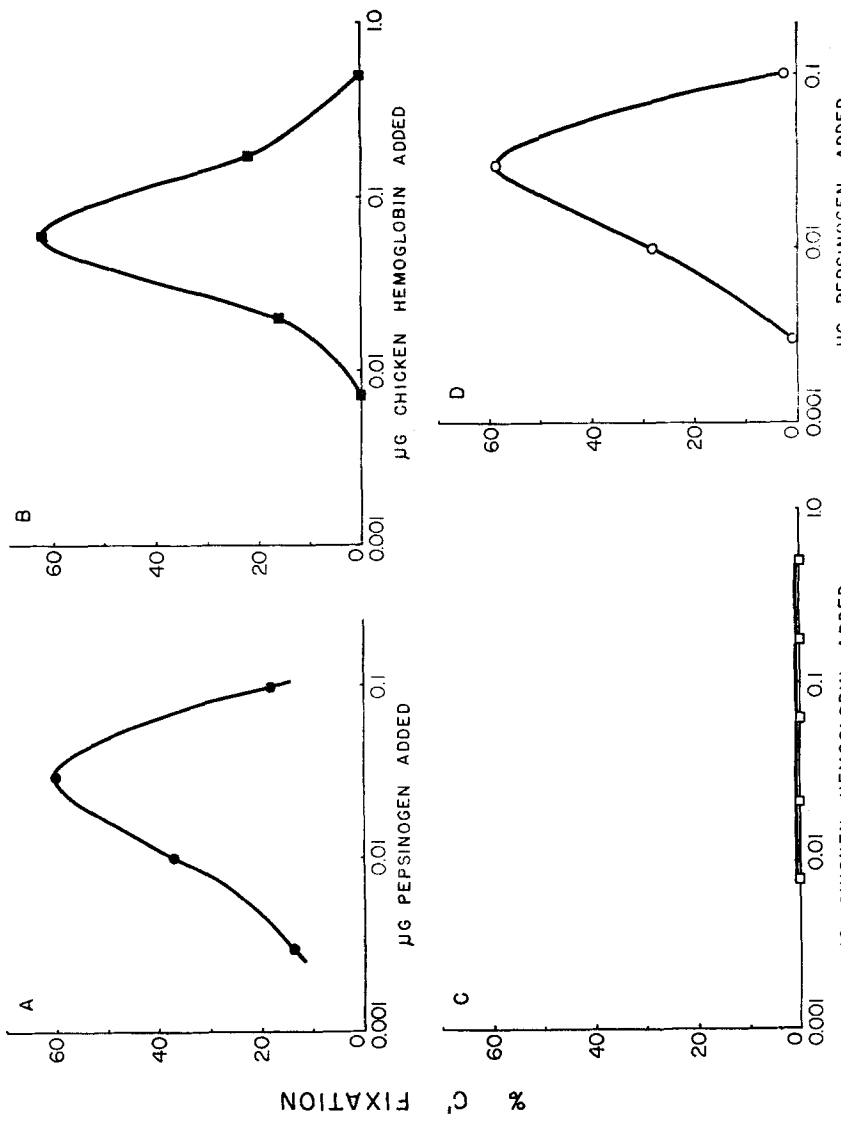

FIG. 6. The effect of a minor antigen-antibody system on the C′ fixation of a major independent antigen-antibody system. *A*, C′ fixation between swine gastric pepsinogen and its rabbit antiserum diluted 1/4000. *B*, C′ fixation between chicken hemoglobin and its rabbit antiserum diluted 1/2000. *C*, lack of C′ fixation between chicken hemoglobin and its antiserum diluted 1/10,000. *D*, C′ fixation between an artificial heterogeneous mixture of the pepsinogen and hemoglobin systems. The antigen mixture contained the same concentrations of *both* pepsinogen and hemoglobin which gave direct C′ fixation, while the antiserum mixture contained *both* antipepsinogen at a dilution of 1/4000 and antihemoglobin at a dilution of 1/10,000. As the presence of the hemoglobin system in the mixture did *not* effect the C′ fixation of the pepsinogen system, the abscissa gives only the concentration of pepsinogen added. The resulting C′ fixation curve was identical with that given by pepsinogen alone (*A*).

geneous state. However, quantitative immunochemical studies of the antigenic relatedness of ovalbumin (15), and of hemoglobins, lactic dehydrogenases (H4 and M4), triosphosphate dehydrogenases, and glutamic dehydrogenases (16) have shown, even with cross-reactions detected at a 1/1000 dilution of antiserum

TABLE II

Biological Response of Acutely Parathyroidectomized Rats to Tumor Extracts

Preparation	No.	Mean plasma calcium
		mg/100 ml ± SE
Control	5	6.38 ± 0.41
15 units, U.S.P parathyroid hormone	5	7.80 ± 0.41*
Tumor extracts (Nos. 1, 2 and 4)	14	7.54 ± 0.24*

* $P < 0.05$, greater than control. Control solution contained reagents without the tissue proteins.

TABLE III

Immunoassay Data from 6 Cases of Hyperparathyroidism Associated with Parathyroid Hormone-Producing Neoplasms

Case No.	Amount of parathyroid hormone measured by immunoassay		
	Tumor tissue		Non-tumor tissue in which no hormone was detected
	Primary	Metastatic	
	μg/gm	*μg/gm*	
1	13 (1.1)*		Kidney
2	10 (1.2)	39 (1.2)	Lung
3	6 (1.1)	0.9 (1.3)	Liver
			Kidney
			Adrenal
4	11 (1.1)		
5	2 (1.2)		
6	6 (1.1)	1.1 (1.2)	Liver
Tumors without hypercalcemia‡	None detected		

* The number in parenthesis, when multiplied by and divided into the estimate of hormone content, gives the limits of the standard error.

‡ Carcinomas of pancreas (two), lung, colon (two), breast, neuroblastoma.

where the homologous C′ fixation is obtained at 1/20,000, that C′ fixation at their respective equivalence zones was obtained with similar quantities of antigen. As these data indicate that the error involved in estimation of a cross-reacting antigen in the zones of antibody excess and equivalence may be small, we considered it meaningful to estimate the hormone content of these hypercalcemic tumors by immunoassay (5).

The immunoassay values for the human tumor tissues are given in Table III in terms of μg of the highly purified bovine reference standard, $3.5 \overset{\times}{\div} 1.18$ units/μg, assuming an equal efficiency of extraction for both parathyroid glands and tumors. The estimates of biological activity in the extracts examined (Table II) were consistent with the immunoassays.

DISCUSSION

The conclusion reached in this paper that a constituent of certain human tumors associated with hypercalcemia is closely similar to, probably identical with, parathyroid hormone is based largely on immunological evidence. The validity of this evidence depends on the specificity and homogeneity of the antiparathyroid hormone used.

With respect to the specificity of the antibody, it had been shown previously that the solution of a washed immune precipitate formed with purified parathyroid hormone contained the biological activity characteristic of the hormone (13). In addition, immunoassays of numerous parathyroid gland extracts covering a wide range of specific activities agreed well with biological assays of the same extracts (5).

The homogeneity of the antiserum was supported first by double diffusion experiments in which crude parathyroid gland extracts as well as the purified hormone gave only one band of precipitation even with concentrated antiserum (5). Secondly, both crude and purified parathyroid hormone preparations gave only a single C′ fixation curve with the antiserum over a wide range of antigen concentrations, even in far antigen excess. If a contaminating antigen-antibody system were present, a second C′ fixation curve would probably have been observed (17). Thirdly, the excellent correlation of immunoassays of parathyroid hormone over a wide range of specific activities with biological assays decreases the likelihood of a contaminating antigen-antibody system. Finally, bovine parathyroid hormone purified in our laboratory by two different methods, and a sample prepared in another laboratory by Dr. Gerald D. Aurbach (13), all gave the same C′ fixation curves.

From the foregoing argument we assume that the antigen found in certain human tumors and in human parathyroid glands is human parathyroid hormone. The increased anti-bovine parathyroid hormone required to visualize the human antigen by C′ fixation (1/100 or 1/200 compared with 1/1500 for the bovine hormone) is probably a function of conformational dissimilarity between the human and bovine hormones. Evidence for antigenic relatedness, by these same criteria, has been shown previously in 5 different antigen-antibody systems (16).

However, the possibility could be considered that the 7 to 13 per cent cross-reaction is in reality a second antigen-antibody system, representing a con-

tamination too small in quantity to detect by the procedures used to test for heterogeneity of the antiserum. If this were true, absorption of the antiserum with the contaminating antigen should not decrease the titer to the homologous purified bovine parathyroid hormone as measured by our C′ fixation procedure. To assess this possibility, we prepared a heterogeneous immune system in which a minor antibody was present as a 20 per cent contaminant. The C′ fixation of the major antigen-antibody system, at high antiserum dilution, was unaffected by the contaminating antigen-antibody system (Figs. 6 *A* to 6 *D*). However, absorption of the antiparathyroid hormone with tumor antigen decreased the antibody titer to the purified bovine parathyroid hormone (Figs. 5 *A* to 5 *C*). This consistent 40 to 50 per cent decrease in C′ fixation by the purified hormone with the antiparathyroid hormone after absorption with tumor extracts would be expected to be only of this magnitude since the cross-reaction is only 7 to 13 per cent (16).

The demonstration, with the artificially prepared heterogeneous antiserum, that the C′ fixation of a major system at a high dilution of antiserum is unaffected by the presence of a 20 per cent contaminating system, reinforces the conclusion that the 70 to 100 per cent inhibition by HCl and phenol extracts of human parathyroid glands and tumors represents inhibition of antiparathyroid hormone antibody. In spite of the demonstrated homogeneity, if it were argued that two antibody systems were being measured at this high dilution (1/1000 to 1/1500) of antiserum, then inhibition could be only partial (less than 100 per cent), unless, of course, the tumor extracts contained both parathyroid hormone and a contaminating inhibitor. Furthermore, unpublished data from our laboratory show that inhibition of C′ fixation is only partial when impure antisera are reacted with pure synthetic haptens (18). The reason for the lack of C′ fixing activity in the presence of inhibiting capacity is unknown at this time. The most likely explanation is that the phenol and HCl extraction procedures alter the conformation and molecular weight of the antigen (19, 20).

Studies with the pepsinogen-pepsin immune system (21) and of the DNA immune system (22) have shown that the antigen molecule can be changed from an antigen that fixes C′ directly with antibody to one that will no longer fix C′ directly but still will react with antibody as evidenced by inhibition of C′ fixation. With respect to the pepsinogen-pepsin immune system, these alterations in the antigen are conformational changes (23), while in the DNA system they are a function of molecular weight (22).

Further support for the conclusion that the tumor antigen represents parathyroid hormone follows from the lack of detectable antigen in control tissues from the same patients whose tumors contained antigen and in tumors of the same organs from patients without hypercalcemia (Table III). One additional renal cell carcinoma found to contain the antigen is omitted from the table be-

cause the patient's serum was not analyzed for calcium. Further study will be necessary to determine to what extent this antigen is characteristic of renal cell carcinomas generally.

Production of parathyroid hormone by the tumor tissue rather than accumulation of the hormone in the tumor from another source is supported by the findings in two of the present cases. Serum calcium in case 1 fell from 15.4 to 8.0 mg/100 ml following removal of the tumor, while tumor recurrence, weeks later, was accompanied by return of hypercalcemia. In case 2, careful anatomic examination of the patient's own parathyroid apparatus (2) showed abnormalities indicative of decreased function.

Further confirmation of the results reported here should be forthcoming from measurements of increased hormone concentration in the serum of patients with this hypercalcemic syndrome. We have examined the serum from only one patient, No. 3, by C′ fixation. A small but definite amount of antigen was measured (at an antibody dilution of 1/200), indicating a circulating hormone level in excess of 10 mμg/ml. To date we have not detected hormone in the serum of any normal person by this method, which is as expected in view of the preliminary data obtained by the radioimmunoassay method of Berson *et al.* (24), showing not more than 0.1 to 1.0 mμg/ml in normal human plasma, a concentration too low to be detected by the C′ fixation assay method as it is presently conducted.

Because of the large mass of the hypercalcemic neoplasms and the relatively high concentration of hormone in them, it may be possible to use them as source material for the isolation and further characterization of human parathyroid hormone.

SUMMARY

1. Immunochemical cross-reactivity, but not identity, has been demonstrated between bovine parathyroid hormone and an antigen in biologically active extracts of human parathyroid tissue by quantitative C′ fixation and C′ fixation inhibition.

2. An antigen that fixes C′ with rabbit antibody to bovine parathyroid hormone has been found in urea extracts of six human non-parathyroid neoplasms associated with a hypercalcemic syndrome mimicking primary hyperparathyroidism. Comparable extracts of control tissues and other tumors were serologically negative.

3. It is concluded that the tumor antigen is parathyroid hormone or a very closely related protein, and that its production by these neoplasms was the cause of the hypercalcemic syndrome in these six patients.

The authors thank the following for their collaboration in this study by submitting specimens for immunoassay: Dr. James B. Lee, Dr. Morton F. Goldberg, Dr. Robert F. Bradley and Dr. Charles Brown, Dr. George L. Cohn, Dr. Robert W. Carey, Dr. Lloyd H. Smith, Jr.

and Dr. Sanford I. Roth, and Dr. Robert Goldman. We also thank Miss Elaine Collura and Mrs. Geraldine Caslowitz for technical assistance, Mrs. Elizabeth A. Moore for statistical work, and Mrs. Joan C. Carlow for the drawings.

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