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Binding of Parathyroid Hormone to Bovine Kidney-Cortex Plasma Membranes

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1. Plasma membranes were purified from bovine kidney cortex, with a fourfold increase in specific activity of parathyroid hormone-sensitive adenylate cyclase over that in the crude homogenate. The membranes were characterized by enzyme studies. 2. Parathyroid hormone was labelled with ^{125}I by an enzymic method and the labelled hormone shown to bind to the plasma membranes and to be specifically displaced by unlabelled hormone. Parathyroid hormone labelled by the chloramine-T procedure showed no specific binding. ^{75}Se-labelled human parathyroid hormone, prepared in cell culture, also bound to the membranes. 3. Parathyroid hormone was shown to retain biological activity after iodination by the enzymic method, but no detectable activity remained after chloramine-T treatment. 4. High concentration of pig insulin inhibited binding of labelled parathyroid hormone to plasma membranes and partially inhibited the hormone-sensitive adenylate cyclase activity in a crude kidney-cortex preparation. 5. EDTA enhanced and Ca^{2+} inhibited binding of labelled parathyroid hormone to plasma membranes. 6. Whereas rat kidney homogenates were capable of degrading labelled parathyroid hormone to trichloroacetic acid-soluble fragments, neither crude homogenates nor purified membranes from bovine kidney showed this property. 7. Binding of parathyroid hormone is discussed in relation to metabolism and initial events in hormone action.

Parathyroid hormone acts directly on the kidney to promote phosphate excretion. Chase & Aurbach (1967) showed that the phosphaturia was preceded by a striking increase in 3′:5′-cyclic AMP in the urine. Subsequently parathyroid hormone was found to stimulate directly the activity of adenylate cyclase in washed, particulate preparations of kidney cortex (Chase & Aurbach, 1968), and this response formed the basis of a sensitive biological assay for the hormone developed by Marcus & Aurbach (1969). It therefore seemed likely that discriminating receptors for parathyroid hormone should exist in the plasma membrane of the target cell, as have been found for glucagon (Rodbell *et al.*, 1971), adrenocorticotrophin (Lefkowitz *et al.*, 1970) and insulin (Cuatrecasas, 1971). In the studies of binding of labelled peptide hormones to receptors, great care has been taken to prepare peptides iodinated with ^{125}I which retain biological activity (Rodbell *et al.*, 1971; Lefkowitz *et al.*, 1970). With parathyroid hormone, labelling with ^{125}I by the method of Hunter & Greenwood (1962) would inevitably result in loss of biological activity, after oxidation of the methionine residues by chloramine-T (Potts *et al.*, 1965). In the present paper we give evidence for the existence of a specific receptor for parathyroid hormone in plasma membranes purified from bovine kidney cortex by using two preparations of labelled hormone: (1) ^{125}I-labelled by the enzymic method of Marchalonis (1969); (2) internally labelled with ^{75}Se by incorporation of ^{75}Se-labelled methionine into parathyroid hormone synthesized by human parathyroid cells in culture (Martin *et al.*, 1973).

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Materials and Methods

Chemicals

^{125}I as NaI (15mCi/μg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., as also was α-^{32}P-labelled ATP (500–2000mCi/mmol). Chemicals were of analytical grade and obtained from standard suppliers.

Hormone preparations

The following preparations of parathyroid hormone were used. (1) Highly purified bovine parathyroid hormone, a gift from Dr. D. V. Cohn and Dr. J. W. Hamilton, Veterans' Administration Hospital, Kansas City, Missouri, U.S.A. This was used for all ^{125}I-labelling, and is referred to as preparation PTH-C.

(2) Purified bovine parathyroid hormone purchased from the Wilson Co., Chicago, Ill., U.S.A., and referred to as preparation PTH-W. Its potency was 700 units/mg, assayed against Medical Research Council bovine parathyroid hormone standard in the rat kidney-cortex adenylate cyclase assay (Marcus & Aurbach, 1969).

(3) Parathyroid hormone prepared in the laboratory from bovine glands to the stage of trichloroacetic acid precipitation by the method of Aurbach (1959), and subjected to gel filtration on Sephadex G-100 in 0.15M-ammonium acetate, pH4.7. The peak of biological activity was pooled, freeze-dried and found to have activity of 600 units/mg in the adenylate cyclase assay (Marcus & Aurbach, 1969). It is referred to as preparation PTH-Seph. Other peptide hormones were obtained as follows: pig insulin (Eli Lilly and Co., Indianapolis, Ind., U.S.A., lot no. 818194), antidiuretic hormone (Sandoz A.G. Products Ltd., Basle, Switzerland, synthetic [lysine]-vasopressin), angiotensin (Ciba Ltd., Basle, Switzerland; Hypertensin), bovine growth hormone (National Institutes of Health, NIH-GH-B10), glucagon (Eli Lilly and Co.), salmon calcitonin (synthetic hormone, gift from Dr. St. Guttman, Sandoz A.G., Basle, Switzerland) and synthetic human adrenocorticotrophin (gift from Dr. Gy. Fekete, Chemical Works of Gideon Richter, Budapest, Hungary).

Preparation of bovine kidney-cortex plasma membranes

Kidney-cortex plasma membranes were prepared by a modification of the method of Neville (1960). Bovine kidneys were obtained from animals immediately after slaughter and taken to the laboratory on ice. All subsequent procedures were carried out at 0°C. The perirenal fat and capsule were removed, then the kidney was cut into 3cm sections and the cortex, dissected carefully from the medulla with scissors, was finely minced and homogenized with a rotor blade in the 500ml container of a Sorvall Omnimix. Initially the homogenizer was calibrated for speed and time of homogenization to obtain optimum conditions for preparation of plasma membranes by taking small samples from the homogenate at 30s intervals at different speeds and examining them by phase-contrast microscopy by using a green filter. For homogenizations and all subsequent dilutions, the solution used was 50mM-Tris-HCl, pH7.4, containing 0.25M-sucrose and 5mM-$CaCl_2$. The homogenates were pooled and diluted to approx. 1000ml, followed by filtration through four thicknesses of surgical gauze. The filtrate was centrifuged at 700*g* in the SS34 head of a Sorvall refrigerated centrifuge for 15min. The top, creamy layer was collected and resuspended by two gentle strokes of a loose-fitting Dounce homogenizer. The suspension was re-centrifuged at 700*g*, this procedure being repeated three to four times until a homogeneous creamy precipitate was obtained. This precipitate was loaded on to a sucrose gradient; sucrose of *d* 1.26 (11.0ml) was slowly added with constant agitation to 1.5ml of the precipitate. Samples (2ml) of this mixture of *d* 1.23 were placed in 5.0ml cellulose tubes for the SW 50.1 head of a Beckman preparative ultracentrifuge. Portions (1ml each) of sucrose *d* 1.20, 1.18, 1.16 were layered on top and centrifugation was carried out for 60min at 32000*g*. The plasma membrane fraction was obtained from the *d* 1.18/*d* 1.16 interface. The plasma membranes were washed free of sucrose by centrifugation in 50mM-Tris-HCl, pH7.4, at 4°C in the Sorvall SS34 head at 27000*g* for 30min and were stored in batches at −80°C. The plasma membranes were characterized by enzyme studies and electron microscopy.

Enzyme studies

The activities of four subcellular markers were determined in seven fractions collected from the process of purification. β-Glucuronidase was assayed as the lysosomal marker by using phenolphthalein glucuronide as the substrate and incubation at 37°C for 10h (Bergmeyer, 1965). Glutamate dehydrogenase activity, chosen as a marker for mitochondria, was measured with glutamate and NAD^+ as the substrate and with quinolylhydrazine to react with α-oxoglutarate formed (Bergmeyer, 1965). Glucose 6-phosphatase activity as a microsomal marker was measured by using glucose 6-phosphate as substrate (Bergmeyer, 1965) and by measuring the P_i released by the method of Parvin & Smith (1969). The 5′-nucleotidase, used as a marker for plasma membranes, was assayed by using 5′-AMP as the substrate (Michell & Hawthorne, 1965) and by measuring P_i released by the method of Parvin & Smith (1969).

Adenylate cyclase activity was assayed in all fractions by measuring the formation of ^{32}P-labelled 3′:5′-cyclic AMP from α-^{32}P-labelled ATP. The incubations (total volume of 80μl) contained 50mM-Tris-HCl (pH7.4), 1.0mM-[α-^{32}P]ATP (specific radioactivity 20c.p.m./pmol), 1mM-cyclic AMP or 4mM-theophylline, 0.13% bovine serum albumin (Commonwealth Serum Laboratories, Parkville, Vic., Australia), 30mM-KCl, 4.5mM-$MgCl_2$, 2.5mM-phosphoenolpyruvate, pyruvate kinase (1.5 units, Sigma Chemical Co., St. Louis, Mo., U.S.A.) and a sample of the subcellular fraction to be tested. After a 10min incubation the reaction was stopped by adding 100μl of 40mM-ATP and boiling for 3min. The cyclic AMP was purified on neutral alumina columns (Ramachandran, 1971; White & Zenser, 1971) and samples were counted for radioactivity in a Packard liquid-scintillation spectrometer. The protein concentration of the fractions was measured by the method of Lowry *et al.*, (1951) with bovine serum albumin as the standard.

Labelling of parathyroid hormone

Lactoperoxidase method (*Marchalonis*, 1969). To 5μg of preparation PTH-C in 5μl of 0.01M-acetic

acid were added 10μl of 0.1M-phosphate buffer, pH7.4 (KH_2PO_4, 13.6g/l; K_2HPO_4, 17.4g/l), 1 or 2mCi of $Na^{125}I$ in 10 or 20μl, followed by 1μg of lactoperoxidase in 25μl of 0.1M-phosphate buffer. The lactoperoxidase was prepared from skim milk by Dr. J. T. Bellair (Prince Henry's Hospital Medical Research Centre, Victoria, Australia) by the method of Hogg & Jago (1970). After addition of 10μl of 0.8mM-H_2O_2, the polystyrene vial was capped and incubated for 10min at 37°C. The reaction was stopped by the addition of 0.5ml of 0.1M-phosphate buffer containing 0.05M-2-mercaptoethanol, and the vial contents were immediately transferred to a tube containing 5mg of microfine silica (Quso G32; Philadelphia Quartz Co., Philadelphia, Pa., U.S.A.). The hormone was bound and eluted from Quso G32 as described by Yalow & Berson (1966) and the final eluate was stored in 100μl portions. These preparations were further purified the day before use by gel filtration on a column (1cm×17cm) of Biogel P60 in 50mM-ammonium acetate buffer, pH4.0, containing 1% bovine serum albumin. A single peak of radioactivity was obtained and the fractions used in binding studies were from the peak tube and the first three tubes on the descending arm of the peak.

Chloramine-T method (Hunter & Greenwood, 1962). To 5μg of preparation PTH-C in 5μl of 0.01M-acetic acid was added 10μl of 0.3M-phosphate buffer, pH7.4 (KH_2PO_4, 40.2g/l; K_2HPO_4, 52.2g/l), followed by 1–2mCi of $Na^{125}I$. Chloramine-T (70μg) was added in 20μl of 0.3M-phosphate buffer and the contents of the reaction vessel were mixed for 20–30s followed by the addition of 240μg of sodium metabisulphite in 100μl of 0.3M-phosphate buffer. Subsequent purification was as described for the iodination with lactoperoxidase.

Internal labelling with ^{75}Se. Monolayer cultures of human parathyroid cells were incubated for 48h with ^{75}Se-labelled methionine in methionine-free medium 199. The radioactive medium was collected and subjected to gel filtration on Sephadex G-100 and the radioactive peak corresponding to parathyroid hormone was pooled and freeze-dried. This fraction was used without further purification and therefore consisted only in part of [^{75}Se]parathyroid hormone. The proportion of [^{75}Se]parathyroid hormone in the fractions varied between 30 and 60% of the total radioactivity. Although the specific radioactivity of the [^{75}Se]parathyroid hormone is presumed to be low, it was not possible to assess it accurately as the size of the intracellular methionine pool was not determined. The characterization of this peak is described in detail elsewhere (Martin *et al.*, 1973).

Binding studies

All incubation mixtures were of 150μl unless otherwise indicated. Plasma membranes (100μl containing 100–200μg of total protein) were incubated for 20–30min in polypropylene tubes (Spinco microcentrifuge tubes) with 50μl of ^{125}I-labelled or ^{75}Se-labelled parathyroid hormone. Incubation buffer was 50mM-Tris–HCl, pH7.4, containing 3% bovine serum albumin. Controls used to measure non-specific binding either contained no added plasma membranes, or membranes which had been heated to 65°C for 1h. Unlabelled parathyroid hormone was diluted in incubation buffer and added to the incubation tubes in volumes of 50μl. After the incubation at 30°C the tubes were centrifuged at 12000*g* for 1min in a Spinco microcentrifuge and the supernatants quickly removed by suction. The tips of the tubes containing the tiny pellet were cut off with a heated scalpel blade and counted for radioactivity in a Packard Auto-Gamma scintillation spectrometer.

Results

Characterization of plasma membranes

Analysis of the enzyme markers showed that fraction six collected from the *d* 1.18/*d* 1.16 interface of the discontinuous sucrose gradient contained the greatest 5′-nucleotidase activity and the least activity of the lysosomal and microsomal markers, β-glucuronidase and glucose 6-phosphatase, but some mitochondrial contamination (Table 1). Further, the specific activity of the parathyroid hormone-sensitive adenylate cyclase in this fraction was increased fourfold over that in the crude homogenate (Table 2). No other gradient fraction contained parathyroid hormone-responsive adenylate cyclase activity. Electron microscopy carried out by Dr. K. Yuro of the Department of Zoology, University of Melbourne, Vic., Australia, confirmed that the fraction was rich in plasma membranes; occasional damaged mitochondria were seen. The difficulty of separating mitochondria from plasma membranes in kidney cortex was only partly overcome owing to the close proximity of mitochondria to the plasma membranes in the tubular villi, which are fragile and easily sheared off during the homogenization procedure.

Biological activity of parathyroid hormone after iodination procedures

Table 3 shows the result of biological assay of parathyroid hormone after it had been labelled with radioactive iodine by the lactoperoxidase and by the chloramine-T methods. ^{125}I was diluted with ^{127}I, since relatively large amounts of hormone were required for the assay. No significant loss of biological activity was found in parathyroid hormone treated by the lactoperoxidase method, whereas complete loss of biological activity occurred after the

Table 1. *Activities of enzyme markers*

Enzyme assays were carried out on all of the fractions obtained during centrifugation. Activity of each enzyme in individual fractions was expressed as a percentage of the total activity of that enzyme in all fractions. Assays were carried out in triplicate. Fractions 1 and 2 were too particulate for accurate measurement of glutamate dehydrogenase activity, and so values for the activity of the mitochondrial marker are approximate only.

		Activity (% of total)			
Fraction	Protein (mg/ml)	β-Glucuronidase	Glucose 6-phosphatase	Glutamate dehydrogenase	5'-Nucleotidase
700*g*	27.5	18.3	0.66	—	0.96
Second wash	14.5	24.3	0.66	—	1.51
Final wash	23.0	22.9	0.40	17.34	4.12
d 1.23/*d* 1.20 interface	1.0	21.6	23.28	19.87	30.54
d 1.20/*d* 1.18 interface	1.4	7.5	12.20	25.37	6.86
d 1.18/*d* 1.16 interface	0.6	2.1	11.17	18.39	48.01
Surface of *d* 1.16	0.6	3.5	51.63	19.03	20.58

Table 2. *Adenylate cyclase activity in fractions from preparation of bovine kidney-cortex plasma membranes*

Parathyroid hormone (preparation PTH-Seph) was included where specified (4μg/incubation mixture). Results are expressed as nmol of cyclic AMP generated in 20min of incubation and are means±S.E.M. of three estimations.

		Adenylate cyclase activity	
Fraction	Additions ...	None	Parathyroid hormone
Crude homogenate		0.42±0.04	0.99±0.05
700*g* precipitate (final wash)		0.64±0.03	1.63±0.14
d 1.18/*d* 1.16 gradient interface		0.77±0.03	3.82±0.12

chloramine-T procedure, even when chloramine-T was added after sodium metabisulphite and immediately before separation from the peptide by gel filtration.

Binding of labelled hormone to plasma membranes

Table 4 shows the results of four experiments in which labelled-hormone preparations were incubated with plasma membranes. There was appreciable binding of [^{125}I]parathyroid hormone prepared by the lactoperoxidase method and a non-specific binding of less than 1% of the total counts added. [^{75}Se]-parathyroid hormone also bound to the membranes, but with considerably higher non-specific binding. Table 5 shows that the binding of [^{75}Se]parathyroid hormone could be inhibited by unlabelled parathyroid hormone. When [^{125}I]parathyroid hormone was prepared by the chloramine-T method a very high non-specific binding was obtained and no evidence found for any binding that was specifically displaced by unlabelled parathyroid hormone (Table 6). In the same experiment the plasma membranes bound an appreciable amount of [^{125}I]parathyroid hormone prepared by the lactoperoxidase procedure, and labelled hormone was displaced by unlabelled hormone at concentrations of 50ng/ml. With the three labelled preparations non-specific binding values were identical whether incubations contained no plasma membranes or membranes that had been heated to 65°C for 1h before use. Non-specific binding of [^{125}I]parathyroid hormone prepared by the chloramine-T method varied appreciably between batches of labelled hormone, but was always greater than 3%. In the experiment shown in Table 6 the radioactivity bound by the plasma membranes was slightly greater than the non-specific binding, but no competition with unlabelled hormone was observed. With some batches of [^{125}I]parathyroid hormone (chloramine-T) there was no net binding of radioactivity by membranes.

Specificity of binding

[Lysine]-vasopressin, angiotensin, adrenocorticotrophin, bovine growth hormone, calcitonin and glucagon did not compete with [^{75}Se]parathyroid hormone or with [^{125}I]parathyroid hormone for

Table 3. *Biological assay of iodine-labelled parathyroid hormone*

Samples of parathyroid hormone (preparation PTH-W) were incubated under the conditions of the chloramine-T and the lactoperoxidase iodination procedures and appropriate controls were carried out. ^{125}I (15mCi/μg) was diluted with $Na^{127}I$ to a specific radioactivity of 10000c.p.m./μg of NaI and used in the incubations. All incubations (samples 1–5) contained 40μg of preparation PTH-W (700 units/mg); at the end of each incubation the volume of each was made up to 200μl with 1% bovine serum albumin in 50mM-Tris–HCl, pH7.0. Other details of the treatment of samples are as follows: (1) chloramine-T iodination as described in the Materials and Methods section, but with 400ng of NaI; (2) as for (1) but sodium metabisulphite was added before chloramine-T; (3) lactoperoxidase iodination as described in the Materials and Methods section, but with 400ng of NaI; (4) as for (3) but omitting the incubation at 37°C for 10min; (5) as for (3) but omitting lactoperoxidase and H_2O_2. At the completion of each iodination or control incubation, each sample was applied immediately to a column (0.5cm×12cm) of Biogel P2 in 0.1M-formic acid. The material eluted at the void volume was collected, freeze-dried, redissolved in 100μl of 0.1M-acetic acid and assayed in the rat kidney-cortex adenylate cyclase bioassay for parathyroid hormone (Marcus & Aurbach, 1969). The recovery of iodinated parathyroid hormone through the column procedure was 70%. No iodination of protein was achieved in incubations 2 and 3, but the same recovery of parathyroid hormone has been assumed. Each sample was assayed at two doses of parathyroid hormone and results are shown also for Medical Research Council parathyroid hormone bioassay standard (67/342). Values are means±S.E.M. of three measurements.

	Adenylate cyclase activity (pmol of cyclic AMP/10min)
Control (no added parathyroid hormone)	22.8±1.9
Sample 1 (2.5μg)	24.9±3.1
(5.0μg)	20.0±4.2
Sample 2 (2.5μg)	20.8±5.1
(5.0μg)	21.2±1.2
Sample 3 (2.5μg)	61.2±6.4
(5.0μg)	87.9±4.2
Sample 4 (2.5μg)	58.1±4.2
(5.0μg)	90.8±6.1
Sample 5 (2.5μg)	65.2±7.1
(5.0μg)	94.2±6.8
Medical Research Council bioassay standard	
(0.2 unit)	41.2±9.1
(1.0 unit)	51.5±1.2
(2.5 units)	95.1±4.8

Table 4. *Binding of labelled parathyroid hormone to kidney-cortex plasma membranes*

Incubations were at 30°C for 20min. Values shown are from four experiments with different preparations of plasma membranes. Specific radioactivities of [^{125}I]parathyroid hormone were 50mCi/mg (Expt. 1) and 80mCi/mg (Expt. 2). Specific radioactivities of [^{75}Se]parathyroid hormone preparations were not assessed. Amounts of radioactivity added in Expts. 1 and 2 were 30000c.p.m. and in Expts. 3 and 4 were 6000c.p.m. Results are expressed as the percentage of the added radioactivity bound to membranes (means±S.E.M. of four observations).

		Binding (% of added radioactivity)	
Expt. no.	Label	Non-specific binding	Binding to plasma membranes
1	[^{125}I]Parathyroid hormone (lactoperoxidase)	0.65±0.03	5.71±0.85
2	[^{125}I]Parathyroid hormone (lactoperoxidase)	1.00±0.14	10.98±0.48
3	[^{75}Se]Parathyroid hormone	6.21±0.28	12.34±0.50
4	[^{75}Se]Parathyroid hormone	3.89±0.29	8.53±0.76

Table 5. *Binding of [^{75}Se]parathyroid hormone to plasma membranes*

Results of two experiments with two different plasma membrane preparations are shown. Incubations were carried out for 20min; incubation volumes were 150μl, containing 100μg of plasma membranes and 5000c.p.m. (Expt. 1) and 2000c.p.m. (Expt. 2) of [^{75}Se]parathyroid hormone. Preparation PTH-W (8μg) was added to half the tubes. Non-specific binding values were 540±25c.p.m. (Expt. 1) and 88±5c.p.m. (Expt. 2). These have been subtracted to give the final values. Results are expressed as c.p.m. bound to membranes (means±S.E.M. of four observations).

Expt. no.	Additions ...	Binding (c.p.m.) None	Binding (c.p.m.) Preparation PTH-W
1		616.0±81.9	356.7±34.9 (P<0.001)
2		142.2±20.5	31.3±14.3 (P<0.001)

Table 6. *Binding of labelled parathyroid hormone to kidney-cortex plasma membranes: comparison of [^{125}I]parathyroid hormone prepared by chloramine-T and lactoperoxidase methods*

Incubations were carried out for 30min and contained 120μg of plasma membrane protein, 6×10^4c.p.m. of each labelled hormone preparation and amounts of preparation PTH-C as specified. Specific radioactivities were 150mCi/mg (chloramine-T) and 30mCi/mg (lactoperoxidase). Results are expressed as c.p.m. bound and are means±S.E.M. of four observations.

	Binding (c.p.m.) [^{125}I]Parathyroid hormone (chloramine-T)	Binding (c.p.m.) [^{125}I]Parathyroid hormone (lactoperoxidase)
Non-specific binding	2902±364	926± 76
Preparation PTH-C Nil	3379±254	4336±201
20ng	3962±425	3918±114
50ng	3817±737	3112±230
100ng	3493±387	2882± 70

Table 7. *Specificity of binding of parathyroid hormone to kidney-cortex plasma membranes*

Incubations (200μl final volume) contained 100μg of plasma-membrane protein and either 6×10^3c.p.m. of [^{75}Se]parathyroid hormone or 4×10^4c.p.m. of [^{125}I]parathyroid hormone (specific radioactivity 150mCi/mg). Unlabelled peptides were added in the amounts specified. Incubations were carried out for 20min at 30°C. Non-specific binding values were 171±18c.p.m. and 384±14c.p.m. for the ^{125}I- and ^{75}Se-labelled parathyroid hormone respectively. Results are means±S.E.M. of four values.

Addition	Amount added	Binding (c.p.m.) [^{125}I]Parathyroid hormone	Binding (c.p.m.) [^{75}Se]Parathyroid hormone
Nil		3112±461	918±104
Preparation PTH-C	12ng	2294±400	632± 86
[Lysine]-vasopressin	1μg	3073±149	919± 29
Bovine growth hormone	1μg	2848± 7	1075± 87
Glucagon	1μg	3137±357	992± 68
Adrenocorticotrophic hormone	1μg	2815±269	950± 44
Angiotensin	1μg		1030±160
Insulin	100ng	3048± 46	
	1μg	2277±233	703± 24
Salmon calcitonin	100ng	3497±147	

Table 8. *Effect of insulin on parathyroid hormone-stimulated adenylate cyclase activity*

A crude preparation of rat kidney cortex was made by the method of Marcus & Aurbach (1971), and the adenylate cyclase assay was carried out as described in the Materials and Methods section. Incubations contained 280 μg of protein from crude membranes. Preparation PTH-Seph (600 units/mg) was used at 2.5 μg/incubation mixture and pig insulin at 5 μg/incubation mixture. Final concentration of glucose was 5 mM. Results are means ± S.E.M. of four values.

	Adenylate cyclase activity (pmol cyclic AMP formed/10 min)
Control	25.6 ± 2.6
Parathyroid hormone	84.3 ± 10.9
Parathyroid hormone + insulin	57.4 ± 9.3*
Parathyroid hormone + insulin + glucose	50.0 ± 2.1*
Parathyroid hormone + glucose	70.0 ± 7.8
Insulin	26.7 ± 1.8
Glucose	26.8 ± 2.6
Insulin + glucose	25.6 ± 2.8

* Differs significantly from parathyroid hormone-stimulated value ($P < 0.001$).

Table 9. *Effect of EDTA (disodium salt) and Ca^{2+} on the binding of [^{125}I]parathyroid hormone to plasma membranes*

Incubations (200 μl final volume) contained 100 μg of plasma membrane protein and were carried out for 30 min. All tubes contained 5×10^4 c.p.m. of [^{125}I]-parathyroid hormone (100 mCi/mg) prepared by the lactoperoxidase method and half the tubes contained unlabelled preparation PTH-C as indicated. EDTA (disodium salt) and Ca^{2+} were included as specified. Non-specific binding of 220 ± 15 c.p.m. was the same for all groups. Results are means ± S.E.M. of three values.

	[^{125}I]Parathyroid hormone bound (c.p.m.)	
	Basal	Preparation PTH-C (10 ng)
Control	2860 ± 264	2353 ± 110
EDTA (1 mM)	4450 ± 30	2793 ± 254
Ca^{2+} (1 mM)	1570 ± 196	1236 ± 26
Ca^{2+} (5 mM)	1333 ± 312	743 ± 59

binding to the plasma membranes (Table 7); however, insulin in microgram amounts did cause displacement. Since insulin was able to affect the binding of labelled parathyroid hormone the effect of insulin on parathyroid hormone-stimulated adenylate cyclase activity was studied. With a crude, particulate preparation of rat kidney cortex (Marcus & Aurbach, 1971), insulin was found to inhibit partially the adenylate cyclase response to parathyroid hormone (Table 8). Glucose had no effect, and neither insulin nor glucose affected basal activity.

Effect of Ca^{2+} and EDTA

Table 9 shows the binding of [^{125}I]parathyroid hormone and its displacement in the presence of 1 mM-EDTA and of 1 mM- and 5 mM-Ca^{2+}. EDTA enhanced binding whereas Ca^{2+} was inhibitory at a concentration of 1 mM and to a greater extent at 5 mM concentration.

Metabolism of parathyroid hormone

Crude homogenates of rat kidney caused rapid breakdown of [^{125}I]parathyroid hormone into trichloroacetic acid-soluble fragments (Table 10) whereas homogenates of bovine kidney had virtually no effect. Similarly, the purified fractions from bovine kidney had virtually no effect on the labelled parathyroid hormone.

Discussion

Since tracers for the study of peptide hormone–receptor interactions need to be biologically active (Rodbell *et al.*, 1971; Cuatrecasas, 1971; Freychet *et al.*, 1971), it was not surprising that parathyroid hormone labelled in the presence of the strong oxidizing agent chloramine-T did not bind to membranes. It is well recognized that parathyroid hormone loses biological activity after oxidation (Potts *et al.*, 1965). On the other hand, when parathyroid hormone was labelled enzymically in the presence of a low concentration of H_2O_2 (approx. 0.1 mM), the [^{125}I]parathyroid hormone bound to plasma membranes and was displaced by low concentrations of unlabelled hormone. It seems likely that the hormone substantially retains its biological activity under these conditions and this is supported by the results of biological assay, in which parathyroid hormone labelled enzymically with ^{127}I suffered no detectable

Table 10. *Metabolism of labelled parathyroid hormone by ox kidney-cortex fractions and rat kidney-cortex homogenate*

Incubation mixtures (vol. 200μl) contained 4×10^4c.p.m. of [^{125}I]parathyroid hormone (80mCi/mg) prepared by the lactoperoxidase method and the kidney fractions in the concentrations indicated. The intact labelled hormone was measured by precipitation with 1ml of cold 10% (w/v) trichloroacetic acid at 0, 10 and 20min. Samples were centrifuged at 3000rev./min for 10min in an MSE centrifuge, washed twice with 1ml of 10% trichloroacetic acid and their radioactivity was counted. Fractions 1, crude ox kidney-cortex homogenate; 2, from *d* 1.20/*d* 1.18 interface of gradient; 3, plasma-membrane fraction; 4, from the surface of *d* 1.16 layer in gradient. Results are expressed as % degradation of [^{125}I]parathyroid hormone to trichloroacetic acid-soluble fragments and are means±S.E.M. of three observations.

Fraction no.	% degradation of [^{125}I]parathyroid hormone			Protein
	0min	10min	20min	(mg/ml)
1	0	0	13.0±2.0	5.7
2	0	6.0±1.0	7.0±2.7	1.5
3	0	0	6.0±2.0	1.65
4	0	2.5±1.0	4.0±1.5	1.25
Rat kidney-cortex homogenate	0	32.0±8.5	41.3±5.4	2.1

loss of activity. Absolute proof of this would require separation of labelled from unlabelled hormone in the mixture obtained from enzymic iodination and bioassay of the two separately. Although we have no information on the biological activity of the various [^{75}Se]parathyroid hormone preparations used, they were not exposed to oxidizing conditions during biosynthesis and subsequent partial purification. The binding of [^{75}Se]parathyroid hormone and specific displacement by unlabelled hormone is further evidence for the existence of a specific receptor for parathyroid hormone in the kidney-cortex plasma membranes.

A new approach to the problem of obtaining a biologically active, labelled parathyroid hormone has been provided by the experiments of Zull & Repke (1972*a*), who treated purified parathyroid hormone with high-specific-radioactivity tritiated methyl ester of acetamidate and obtained a labelled product capable of stimulating kidney-cortex adenylate cyclase activity. They studied tissue uptake and metabolism of parathyroid hormone by using this tritiated derivative and showed that *in vivo* the kidney very actively accumulated and degraded parathyroid hormone after its injection (Zull & Repke, 1972*b*), confirming earlier findings from our laboratories in experiments with [^{125}I]parathyroid hormone, of presumably little biological activity (Martin *et al.*, 1969; de Luise *et al.*, 1970; de Kretser *et al.*, 1970). It is particularly notable that oxidation of tritiated parathyroid hormone, although impairing its biological activity, did not decrease its uptake by the kidney *in vivo* (Zull & Repke, 1972*b*). The results of Zull & Repke (1972*b*), together with those reported here, indicate that reduced methionine residues and retention of biological activity are not necessary for initial uptake of parathyroid hormone by the rat kidney *in vivo* and metabolism *in vitro*; however, biological activity is essential for binding to plasma membranes *in vitro*. Since the latter may signify the initial step in the reaction with the target cell, leading to activation of the membrane-bound adenylate cyclase, it is apparent that binding of peptide hormone to the receptor and proteolytic degradation of the hormone, processes frequently located together on the plasma membrane (Freychet *et al.*, 1972), may be separate entities. In relation to this, it was surprising to find that bovine kidney fractions had little capacity to metabolize labelled parathyroid hormone, whereas rat kidney slices, homogenates and subcellular fractions have been shown to degrade the labelled hormone efficiently (Orimo & Fujita, 1966; Martin *et al.*, 1969; Zull & Repke, 1972*b*; Fang & Tashjian, 1972). This provides a further example of species difference in the metabolism of peptide hormones, such as that shown for calcitonin (de Luise *et al.*, 1972), and indicates the need for studies of parathyroid hormone metabolism in species other than the rat.

Evidence for a link between hormone binding *in vitro* and activation of adenylate cyclase is provided by the experiments with insulin, EDTA and Ca^{2+}. Insulin inhibited [^{125}I]parathyroid hormone binding and also inhibited the response of adenylate cyclase to parathyroid hormone. The fact that insulin has been found to inhibit glucagon and adrenaline-sensitive adenylate cyclase activity (Illiano & Cuatrecasas, 1971) may indicate a general effect of

insulin on membranes, perhaps leading to configurational changes and so affecting binding and responsiveness of adenylate cyclase. Pohl *et al.* (1971) found that 1mM-EDTA enhanced the response of adenylate cyclase to glucagon in liver, and also markedly increased the dissociation of bound glucagon from liver membranes. In the experiments reported here 1mM-EDTA enhanced the capacity of kidney-cortex plasma membranes to bind labelled parathyroid hormone and also increased the dissociation of the peptide from the membranes. It is well documented that Ca^{2+} inhibits parathyroid hormone-stimulated adenylate cyclase (Marcus & Aurbach, 1971; Streeto, 1969); Ca^{2+} at concentrations of 1 and 5mM also inhibited binding of parathyroid hormone to the plasma membranes, further suggesting a link between binding of the hormone to the plasma membrane and stimulation of adenylate cyclase.

Investigations of the nature of parathyroid hormone receptors and the factors influencing interaction between hormone and receptor, and also between the receptor and stimulation of adenylate cyclase, will be important in elucidating early events in the action of the hormone; it will be of particular interest to study the relationships between receptor binding and metabolic degradation of parathyroid hormone.

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References

Aurbach, G. D. (1959) *J. Biol. Chem.* **234**, 3179–3181
Bergmeyer, H. U. [ed.] (1965) *Methods of Enzymatic Analysis*, Academic Press, New York
Chase, L. R. & Aurbach, G. D. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 518–525
Chase, L. R. & Aurbach, G. D. (1968) *Science* **159**, 545–548
Cuatrecasas, P. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 1264–1268
de Kretser, D. M., Martin, T. J. & Melick, R. A. (1970) *J. Endocrinol.* **46**, 507–510
de Luise, M., Martin, T. J. & Melick, R. A. (1970) *J. Endocrinol.* **48**, 173–179
de Luise, M., Martin, T. J., Greenberg, P. B. & Michelangeli, V. (1972) *J. Endocrinol.* **53**, 475–482
Fang, V. S. & Tashjian, A. H., Jr. (1972) *Endocrinology* **90**, 1177–1184
Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) *Biochem. Biophys. Res. Commun.* **43**, 400–407
Freychet, P., Kahn, R., Roth, J. & Neville, D. M., Jr. (1972) *J. Biol. Chem.* **247**, 3953–3961
Hogg, D. McC. & Jago, G. R. (1970) *Biochem. J.* **117**, 779–790
Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
Illiano, G. & Cuatrecasas, P. (1971) *Science* **175**, 906–908
Lefkowitz, R. J., Roth, J., Pricer, W. & Pastan, I. (1970) *Proc. Nat. Acad. Sci. U.S.* **65**, 745–752
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299–305
Marcus, R. & Aurbach, G. D. (1969) *Endocrinology* **85**, 801–810
Marcus, R. & Aurbach, G. D. (1971) *Biochim. Biophys. Acta* **242**, 410–421
Martin, T. J., Melick, R. A. & de Luise, M. (1969) *Biochem. J.* **111**, 509–514
Martin, T. J., Greenberg, P. B. & Michelangeli, V. (1973) *Clin. Sci.* **44**, 1–8
Michell, R. H. & Hawthorne, J. N. (1965) *Biochem. Biophys. Res. Commun.* **21**, 333–338
Neville, D. M., Jr. (1960) *J. Biophys. Biochem. Cytol.* **8**, 413–422
Orimo, H. & Fujita, T. (1966) *Endocrinology* **78**, 884–886
Parvin, R. & Smith, R. A. (1969) *Anal. Biochem.* **27**, 65–72
Pohl, S. L., Birnbaumer, L. & Rodbell, M. (1971) *J. Biol. Chem.* **246**, 1849–1856
Potts, J. T., Jr., Aurbach, G. D., Sherwood, L. M. & Sandoval, A. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 1743–1751
Ramachandran, J. (1971) *Anal. Biochem.* **43**, 227–239
Rodbell, M., Krans, M. J., Pohl, S. L. & Birnbaumer, L. (1971) *J. Biol. Chem.* **246**, 1861–1871
Streeto, J. M. (1969) *Metabolism* **18**, 968–973
White, A. A. & Zenser, T. V. (1971) *Anal. Biochem.* **41**, 372–396
Yalow, R. S. & Berson, S. A. (1966) *Nature (London)* **212**, 357–358
Zull, J. E. & Repke, D. W. (1972*a*) *J. Biol. Chem.* **247**, 2183–2188
Zull, J. E. & Repke, D. W. (1972*b*) *J. Biol. Chem.* **247**, 2195–2199