Alkylation and Oxidation of Methionine in Bovine Parathyroid Hormone: Effects on Hormonal Activity and Antigenicity*

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Incubation of bovine parathyroid hormone with iodoacetate resulted in a progressive and parallel decline in the calcium-mobilizing and phosphaturic activities of the hormone. Inactivation of the hormone by oxidation with hydrogen peroxide, which is reversed by cysteine, protected against the effect of subsequent treatment with iodoacetate. Analyses of amino acid composition and ultraviolet and fluorescence spectra indicated that both alkylation with iodoacetate and oxidation with peroxide involved only the methionine in the hormone polypeptide. The antigenic activity of the hormone, measured by complement fixation, was not changed qualitatively or quantitatively by alkylation or oxidation, suggesting that these changes in the methionine component had not produced any major alterations in the conformation of the molecule, and demonstrating that the structural requirements for biological and immunological activity are not identical.

The polypeptide hormone of the parathyroid gland can be inactivated by oxidation with hydrogen peroxide and reactivated by incubation with cysteine (Rasmussen 1958; Rasmussen and Craig, 1962a). The similar reversible loss in activity of ACTH following treatment with peroxide has been shown to be caused by a single change in the hormone molecule, oxidation of the methionine residue at position 4 to methionine sulfoxide (Dedman *et al.*, 1961). The importance of methionine for the activity of parathyroid hormone also is supported by the data of Rasmussen and Craig (1962a) showing a correlation between decline in biological activity and increase in methionine sulfoxide content during the controlled oxidation of the hormone. However, the analyses of methionine sulfoxide by Rasmussen and Craig were preceded by alkaline hydrolysis, a procedure that may fail to give accurate estimates of the products of oxidation of methionine (Neumann *et al.*, 1962). In addition, the possibility was not excluded that amino acid residues other than methionine were affected by peroxide.

Utilizing the reaction at acidic *p*H of iodoacetic acid with methionine, which does not occur with methionine sulfoxide (Neumann *et al.*, 1962), we have demonstrated that methionine is the sole amino acid residue in parathyroid hormone that undergoes reversible oxidation-reduction with peroxide and cysteine and that either alkylation or oxidation of the methionine sulfur atom results in the loss of both the calcium-mobilizing and phosphaturic activities of the hormone. We also found that there was no change in the antigenic activity of the polypeptide following alkylation or oxidation of the methionine, indicating that the sites of hormonal and immunological activity on the molecule are not identical and that no major alteration in the conformation of the molecule accompanied these modifications of the methionine component.

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MATERIALS AND METHODS

Parathyroid Hormone Preparations.—In this study partially purified parathyroid hormone was used for preliminary observations of the effects of chemical modifications of the hormone on its biological activity *in vivo*. All critical experiments were repeated with highly purified parathyroid hormone, which was also used for identification of the specific amino acid residues in the hormone polypeptide chain affected by oxidation and alkylation.

Acetone powder of bovine parathyroid tissue (Wilson Laboratories, Chicago, Ill.) was extracted with phenol and partially purified to the step of trichloroacetic acid precipitation by the method of Aurbach (1959). The specific biological activity of this trichloroacetic acid precipitate, partially purified parathyroid hormone, by the assay method of Munson (1961) for calcium-mobilizing activity, was $4300 \times \div 1.25$ units/mg nitrogen (N).[1] Further fractionation of the partially purified parathyroid hormone was achieved by two successive gel filtrations on columns of Sephadex G-50 (Rasmussen and Craig, 1962b). A high degree of purity of the final gel filtration product, highly purified parathyroid hormone, was indicated by the results of three different methods of evaluation.

First, the specific biological activity of the highly purified parathyroid hormone by the assay method of Munson (1961) was $40{,}000 \times \div 1.21$ units/mg N,[1] a value as high or higher than any previously reported by other investigators.

Second, a rabbit antiserum produced against partially purified parathyroid hormone (Tashjian *et al.*, 1962), which gave more than one peak of complement (C′) fixation with impure hormone preparations, gave only a single C′-fixation peak with the highly purified hormone even in far antigen excess. If a contaminat-

[1] The factor following the symbol, $\times \div$, when multiplied by and divided into the potency estimate, gives the limits of the standard error. Protein nitrogen (N) was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Hereafter, where amounts of hormone preparations are given in mg or μg, they denote quantities of dry samples weighed on the Cahn Electrobalance or aliquots thereof. Agreement between actual weight and N analysis × 6.25 was within ±30%.

ing antigen-antibody system had been present, a second C′-fixation curve would probably have been observed (Reichlin *et al.*, 1963). For comparison with the gel filtration product a sample of the partially purified hormone was fractionated further in our laboratory by countercurrent distribution (Aurbach, 1959). Using the anti–partially purified parathyroid hormone known to contain antibodies to contaminating proteins, we obtained superimposable C′-fixation curves with the highly purified hormone, the countercurrent distribution preparation, and with a third sample of highly purified hormone prepared by gel filtration in another laboratory by Dr. Gerald D. Aurbach (Tashjian *et al.*, 1962). In addition, another rabbit antiserum produced against the highly purified hormone was allowed to diffuse in agar for 72 hours, both at 4° and at 20°, against the immunizing antigen in comparison with crude parathyroid gland extracts (300 units/mg N). There was only one band of precipitation between each of the antigens and undiluted antiserum or antiserum concentrated four times by lyophilization. This anti–highly purified parathyroid hormone also gave identical C′-fixation curves with the gel filtration and countercurrent-distribution products. Good agreement between immunoassays (using anti–highly purified parathyroid hormone) and bioassays of parathyroid gland extracts of widely varying potencies was taken as further evidence against serious heterogeneity of the antiserum and, indirectly, of the antigen, highly purified parathyroid hormone, used for immunization (Tashjian *et al.*, 1964).

Third, the vertical starch-gel electrophoresis (Aurbach and Potts, 1964) pattern of the highly purified hormone was compared with patterns obtained with the partially purified hormone and the countercurrent distribution product. The results, published elsewhere (Tashjian *et al.*, 1964) showed a single band for the countercurrent distribution product. The highly purified hormone showed a major band of the same mobility as the countercurrent distribution fraction, but also a very faint band of considerably slower mobility. This less mobile material as well as the major band were each eluted from the gel and tested for hormonal activity (Tashjian *et al.*, 1964) with the Munson (1961) assay method. Biological activity was demonstrated only in the major band. The slower component may represent a minor residual contaminant or an artifact produced during purification by gel filtration. The electrophoretic patterns of these preparations are shown in Figure 1A,B.

The high potency, the lack of immunochemical heterogeneity, and the appearance on starch-gel electrophoresis of only a trace of contamination were considered to indicate a sufficiently high degree of purity of the hormone highly purified by gel filtration for identification of the site of reversible oxidation-reduction in the polypeptide.

Biological Assays.—Calcium-mobilizing and phosphaturic activities were estimated in acutely parathyroidectomized rats by the assay methods of Munson (1961) and Kenny and Munson (1959), respectively. Plasma, obtained by cardiac puncture 5 hours after subcutaneous administration of the test sample, was analyzed for calcium by a semiautomated modification of the method of Munson *et al.* (1955), which is based on titration with EDTA. Spontaneously voided urine was collected for 3 hours (instead of 6 hours as in the original method of Kenny and Munson, 1959) in metabolism cages and was analyzed for inorganic phosphate by the method of Fiske and Subbarow (1925).

Vertical Starch-Gel Electrophoresis.—Vertical starch-gel electrophoresis in 8 M urea and 0.1 N formic acid was

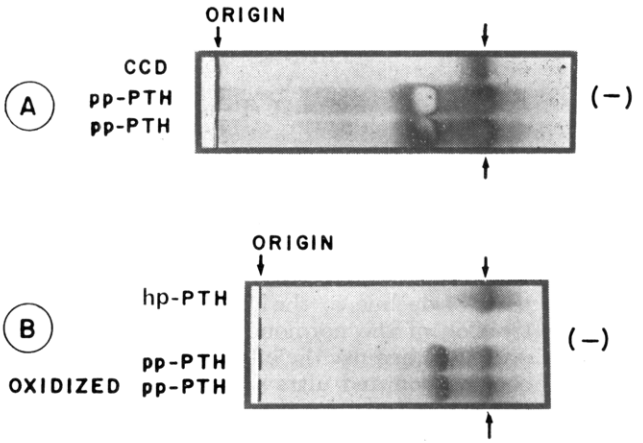

FIG. 1.—Vertical starch-gel electrophoresis of partially purified parathyroid hormone (pp-PTH) and of hormone highly purified by countercurrent distribution (CCD) or gel filtration (hp-PTH). Each channel contained 400 μg of sample in 0.05 ml except the two partially purified parathyroid hormone channels in A that contained 450 μg. Localization of the hormone, shown by the arrows, in this electrophoretic system was accomplished by biological and immunological assays of serial segments of the gel (Tashjian *et al.*, 1964). The mobility of the hormone, shown in B, is unaltered by H_2O_2 oxidation.

performed according to Aurbach and Potts (1964). The samples were subjected to electrophoresis with the cathode at the top of the gel for 18 hours at 25° at a current of 30 ma. The peptides in the gel were stained with 0.0125% Amido Black 10B and 0.0125% Nigrosin in methanol–acetic acid–water (5:1:4).

Alkylation.—Iodoacetic acid, recrystallized three times from petroleum ether, was incubated at 37° with solutions of parathyroid hormone (partially purified or highly purified) at an iodoacetic acid concentration of 0.007 M. Before incubation the *p*H of the reaction mixture was adjusted to the desired value by addition of dilute NaOH or HCl. Following incubation for 1–22 hours, the reaction mixtures were cooled in ice, and the unreacted iodoacetic acid was removed either by dialysis for a total of 3 hours in 8/32 Visking tubing at 4° against two changes of 300 volumes of water adjusted to *p*H 3.8, or by passing the mixture rapidly through a 3.5 × 0.6-cm column of Amberlite IR-45 (OH^- form). In the latter method, which was used to remove iodoacetic acid from all samples to be analyzed for amino acid composition, the effluent solution contained 90% of the original protein but less than 3.8×10^{-4} M iodoacetic acid, the limit of sensitivity of a modified iodine spot test (Feigl, 1958).

Oxidation with Hydrogen Peroxide.—Parathyroid hormone (partially purified or highly purified) was incubated with 0.1 M hydrogen peroxide (H_2O_2) at *p*H 3.8 for 30 minutes at 25°. The reaction was terminated either by the addition of 0.25 mg of catalase (Nutritional Biochemicals Corp.) per ml or, in experiments where amino acid analyses were to be performed, by dilution of the reaction mixture with an equal volume of cold water followed by freezing at −60° and immediate lyophilization.

Reduction of Oxidized Hormone with Cysteine.—Samples previously oxidized with H_2O_2 were reduced by incubation with 0.12 M cysteine hydrochloride (Eastman) for 3 hours at 80°.

Oxidation with Performic Acid.—Samples of highly purified parathyroid hormone (1.5 mg) in 0.05 ml 99% formic acid were oxidized at −6° with performic acid (0.1 ml), following the procedure of Hirs (1956).

Amino Acid Analyses.—The amino acid analyses of

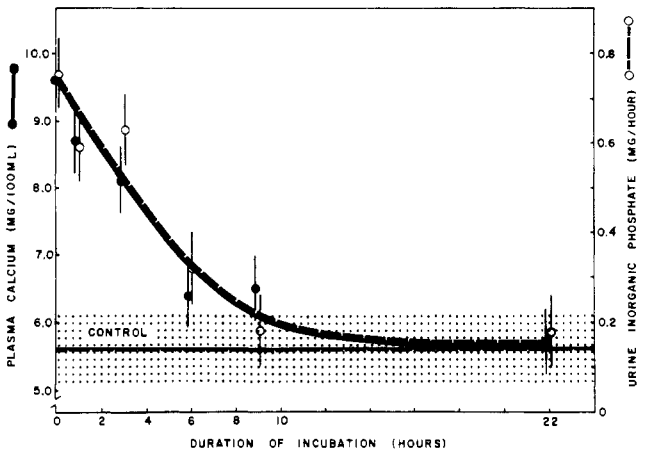

FIG. 2.—Rate of inactivation of partially purified parathyroid hormone (600 μg/ml) by incubation with iodoacetic acid (0.007 M, *p*H 3.8, 37°). Each point represents a mean value from five parathyroidectomized rats. The dose per rat was 0.25 ml. The vertical lines give the standard errors. Control levels for plasma calcium and urine inorganic phosphate are given by the horizontal lines, and the stippled band shows the standard errors of both control means.

hydrolyzed samples of highly purified parathyroid hormone were performed by ion-exchange chromatography (Moore *et al.*, 1958) on a Beckman-Spinco amino acid analyzer equipped for automatic recording (Spackman *et al.*, 1958).

The integration constants used for homoserine, its lactone, and *S*-carboxymethylhomocysteine were, respectively, 19.08, 13.88, and 20.50; and those for the neighboring amino acids, serine, alanine, and histidine were, respectively, 21.81, 21.95, and 21.89.

The molar ratios for the amino acids in parathyroid hormone were calculated by assuming values of 7.00 for lysine, 3.00 for histidine, 2.00 for phenylalanine, and 2.00 for isoleucine.

Ultraviolet and Fluorescence Spectra.—Measurements of the ultraviolet-absorption spectra of both unmodified and alkylated highly purified parathyroid hormone were made with a Cary recording spectrophotometer at *p*H 3.8 and in 0.1 N NaOH. Unreacted iodoacetic acid was removed by the column method (IR-45). A solution containing the same initial concentration of iodoacetic acid was passed through the same column and used as the reagent blank.

Measurements of the fluorescence-emission spectra of the unmodified hormone and the alkylated hormone in 0.1 N NaOH (the same samples used for ultraviolet measurement) were made in an Aminco-Bowman spectrofluorophotometer at activating wavelengths of 280 and 290 mμ.

Immunochemical Studies.—Measurements of the antigenic activity of the parathyroid hormone preparations were made by quantitative C′ fixation as previously described (Tashjian *et al.*, 1964), except that the diluting buffer was isotonic NaCl-Tris containing no added protein. The rabbit antiserum was produced against highly purified parathyroid hormone in the biologically active reduced state.

Statistical Methods.—The results of the biological assays were evaluated by standard statistical procedures for parallel-line assays according to Finney (1952). In experiments comparing the biological effects of untreated and modified hormone preparations, the standard errors and probability (*P*) values were calculated from the pooled residual-variance terms.

RESULTS

Effects of Alkylation and Oxidation on Hormonal Activity

Alkylation of Parathyroid Hormone.—A solution of partially purified parathyroid hormone (1 mg/ml) was divided into aliquots, and each was incubated for 12 hours, either with or without iodoacetic acid, at *p*H values from 2.5 to 10.0. All samples were then dialyzed and tested for effect on plasma calcium of parathyroidectomized rats, as in the Munson (1961) assay method, at a dose level of 160 μg per rat. The results, presented in Table I, indicate that loss of hormonal activity occurred in the aliquots treated with iodoacetic acid at all *p*H levels tested. Measurements of the hormonal activity of partially purified parathyroid hormone incubated with iodoacetic acid at *p*H 3.8 and then tested at a dose seven times (1295 μg/rat) that of a sample incubated in the absence of iodoacetic acid (185 μg/rat) still failed to reveal any significant calcium-mobilizing activity remaining. Subsequent experiments with iodoacetic acid were performed at *p*H 3.8.

TABLE I
INACTIVATION OF PARATHYROID HORMONE BY IODOACETATE AT DIFFERENT *p*H VALUES

Solution	*p*H	Plasma Calcium[a] Incubation with Iodoacetic Acid (mg/100 ml)	Plasma Calcium[a] Incubation without Iodoacetic Acid (mg/100 ml)
Water	5.0[b]	[c]	6.3 ± 0.28
Partially purified parathyroid hormone	2.5	6.1 ± 0.27	10.3 ± 0.75
Partially purified parathyroid hormone	3.8	6.2 ± 0.27	10.2 ± 0.75
Partially purified parathyroid hormone	7.0	6.1 ± 0.27	8.1 ± 0.75
Partially purified parathyroid hormone	10.0	6.6 ± 0.27	9.3 ± 0.75

[a] Mean ± standard error. Five rats per group. [b] *p*H of the water alone. Groups of rats injected with water adjusted to the specified experimental *p*H values from 2.5 to 10.0 showed no significant differences in mean plasma calcium. [c] Solutions of either hormone or water alone plus iodoacetate that were immediately dialyzed without incubation and then injected into assay rats showed, respectively, no loss of hormonal activity (9.6 ± 0.50 mg calcium/100 ml), and no difference from water alone (5.7 ± 0.50 mg calcium/100 ml).

A sample of partially purified parathyroid hormone was dissolved in water and aliquots were incubated with iodoacetic acid for different intervals to determine the time course of inactivation. All mixtures were dialyzed immediately after incubation. Figure 2 shows that there had been essentially complete loss of hormonal activity by the end of 9 hours of incubation. The rates of loss of calcium-mobilizing activity and phosphaturic activity were the same. All subsequent incubations were for 12 hours.

Combined Effects of Peroxide Oxidation and Alkylation.—A solution of partially purified parathyroid hormone (750 μg/ml) was subdivided and aliquots were treated with iodoacetic acid alone, H_2O_2 alone, H_2O_2 followed by cysteine, H_2O_2 followed by iodoacetic acid followed by cysteine, or iodoacetic acid followed by cysteine, as described under Materials and Methods. An untreated sample of hormone was incubated and dialyzed under conditions identical to those used for the experi-

TABLE II
EFFECTS OF OXIDATION, ALKYLATION, AND REDUCTION ON THE HORMONAL ACTIVITIES OF PARTIALLY PURIFIED PARATHYROID HORMONE AND HIGHLY PURIFIED PARATHYROID HORMONE

	Partially Purified Parathyroid Hormone[a]					Highly Purified Parathyroid Hormone[b]		
		Plasma Calcium		Urine Inorganic Phosphate			Plasma Calcium	
Solution	No. of Rats	(mg/100 ml)[c]	(P)[d]	(mg/rat/hr)[e]	(P)[d]	No. of Rats	(mg/100 ml)[c]	(P)[d]
Control	5	5.7 ± 0.50		0.16		4	5.8 ± 0.53	
Intact hormone	5	9.7 ± 0.50	<0.001	0.89	<0.05			
Alkylated hormone	5	6.1 ± 0.50	n.s.[f]	0.18	n.s.			
Oxidized hormone	5	6.3 ± 0.50	n.s.	0.25	n.s.			
Oxidized hormone + reduction	5	11.9 ± 0.50	<0.001	1.01	<0.05	5	7.7 ± 0.48	<0.05
Oxidized hormone + alkylation + reduction	5	10.0 ± 0.50	<0.001	1.03	<0.05	5	8.2 ± 0.48	<0.01
Alkylated hormone + reduction	5	7.5 ± 0.50	<0.05	0.20	n.s.	5	5.8 ± 0.48	n.s.

[a] Each rat received the same dose of partially purified parathyroid hormone (185 μg). [b] Each rat received the same dose of highly purified parathyroid hormone (40 μg). [c] Mean ± standard error. [d] Probability that difference from control mean was due to chance. [e] Mean of two pools of urine from two and three rats each (total of five rats). Since each value represents the mean of only two independent observations, standard errors are not given. [f] Not significantly different ($P > 0.05$) from control mean.

mental samples. All samples were exposed to catalase. The results of biological tests for calcium-mobilizing and phosphaturic activities are shown in Table II. There were clear and parallel losses of both activities in the samples of hormone treated with iodoacetic acid alone or with H_2O_2 alone in comparison with intact untreated hormone. Incubation of oxidized hormone with cysteine fully restored biological activity, while the same treatment of alkylated hormone had little if any effect on activity.[2] It is particularly significant that the sample of hormone oxidized with H_2O_2 before it was exposed to iodoacetic acid could be restored to full activity by subsequent reduction. Confirmatory results with the highly purified hormone are also shown in Table II.

Effects of Alkylation and Oxidation on Chemical Composition and Conformation of Parathyroid Hormone

Amino Acid Analyses.—Quantitative amino acid analyses were carried out to determine the extent of oxidation of methionine in the hormone by H_2O_2 under the conditions used and to detect possible alterations, if any, in other amino acid residues. Because reduction of methionine sulfoxide to methionine (Ray and Koshland, 1960) and other products (Floyd *et al.*, 1963) occurs during acid hydrolysis; and because of the variable recovery of the sulfoxide as well as other residues following alkaline hydrolysis (Neumann *et al.*, 1962), the methionine sulfoxide content of highly purified parathyroid hormone before and after oxidation was determined following the method of Neumann *et al.* (1962). Untreated and oxidized samples of highly purified hormone were first incubated with iodoacetic acid at *p*H 3.8, thereby alkylating methionine, but not methionine sulfoxide residues. The alkylated samples were then oxidized with performic acid to convert methionine sulfoxide to methionine sulfone without altering the alkylated methionine. The sulfone, which, unlike the sulfoxide, is stable to acid hydrolysis, could then be determined chromatographically, the value obtained serving as an indirect measure of the methionine sulfoxide content of the original sample.

The results of the amino acid analyses are given in Table III. The data indicate that methionine alone was alkylated by iodoacetic acid and oxidized by H_2O_2. Amino acid analysis of the untreated sample (column 2) showed 1.08 residues of methionine and 0.19 residue of methionine sulfoxide per molecule. Following performic acid oxidation there were 1.02 residues of methionine sulfone (column 3). Alkylation prior to performic acid oxidation (column 4) produced only the products expected from methionine carboxymethylsulfonium salt hydrolysis (Neumann *et al.*, 1962), namely, methionine (0.29), *S*-carboxymethylhomocysteine (0.37), homoserine (0.16), and homoserine lactone (0.05). The results in column 5, which were essentially the same as those shown in column 3, demonstrated that H_2O_2 oxidation effectively protected against alkylation on subsequent exposure to iodoacetic acid.

Ultraviolet and Fluorescence Spectra.—The data in Table III do not show significant alterations in any amino acid residues other than methionine, with the exception of consistent large losses of tyrosine (columns 3, 4, and 5). In particular, there was no evidence for alkylation of either histidine or lysine. Loss of tyrosine, also noted by Neumann *et al.* (1962), most probably reflected conversion to chlorotyrosine during performic acid oxidation and subsequent hydrolysis. In order to rule out an effect of alkylation per se on the tyrosine residue or on the tryptophane residue (not determined chemically), comparison of the ultraviolet and fluorescence spectra of highly purified parathyroid hormone were made before and after alkylation. Figure 3A shows that in 0.1 N NaOH the ultraviolet-absorption maxima at 282 and 290 mμ were identical for the untreated and alkylated samples. Again, in water at *p*H 3.8, the ultraviolet-absorption maxima of the untreated and alkylated samples were identical at 275 and 290 mμ (Fig. 3B). The molar ratios of tyrosine to tryptophane, calculated from the data in 0.1 N NaOH by the method of Beaven and Holiday (1952), were 1.3 and 1.1 for the untreated and alkylated samples, respectively. Figure 4 shows the fluorescence-emission spectra of highly purified parathyroid hormone in 0.1 N NaOH before and after alkylation. At activation

[2] The apparent partial regeneration of hormonal activity in the alkylated partially purified hormone sample that was reduced by incubation with cysteine was probably owing to the presence of methionine sulfoxides in this preparation prior to alkylation. When this partially purified hormone was reduced *before* exposure to iodoacetic acid, alkylation completely inactivated the sample and no regeneration was seen following a second incubation with cysteine.

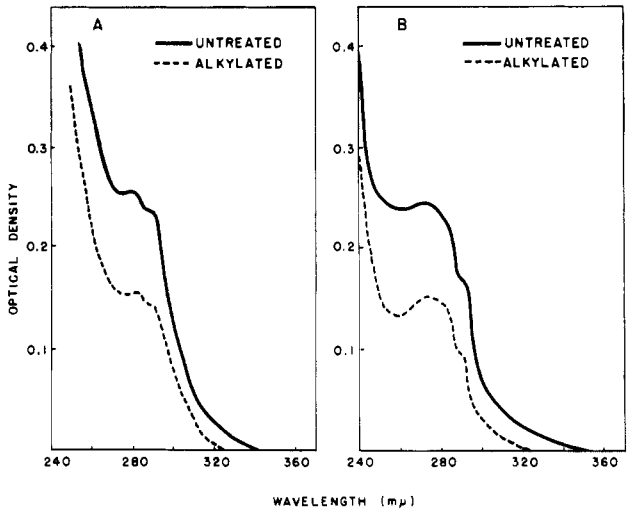

FIG. 3.—Ultraviolet-absorption spectra of untreated and alkylated highly purified parathyroid hormone (350 μg/ml), A, in 0.1 N NaOH, and B, in water adjusted to *p*H 3.8 with dilute HCl. Unreacted iodoacetic acid was removed from the alkylated sample by the IR-45 column method. A sample of iodoacetic acid was processed through the same column and used as a blank. The uniform decrease in optical density seen following alkylation reflects dilution of the polypeptide during separation from iodoacetic acid.

wavelengths of 280 and 290 mμ the emission maxima for both preparations were identical at 355 mμ.

Starch-Gel Electrophoresis.—Figure 1A,B shows the electrophoretic patterns of the two preparations highly purified by countercurrent distribution and gel filtration, and also of the partially purified hormone. The position of the biologically active hormone in the partially purified preparations is shown by the arrows. The mobility of this hormone band following H_2O_2 oxidation (Fig. 1B) appeared to be exactly the same as that of the unoxidized sample in the adjacent channel.

Immunochemical Experiments.—The experiments described indicated that the losses of hormonal activity of the parathyroid polypeptide following treatment with H_2O_2 or iodoacetic acid were both effected through an alteration of methionine. It was not known, however, whether the conversion of the thioether to the sulfoxide or its alkylation caused secondary alterations in the conformation of the molecule that were responsible for the inactivation of the hormone. The availability of an antiserum to highly purified parathyroid hormone in its reduced, hormonally active state offered a means for detecting a possible change in the conformation of the molecule following chemical modification.

C′-fixation curves obtained with anti–highly purified parathyroid hormone and samples of either partially purified or highly purified hormone that were untreated, alkylated, oxidized, or oxidized before exposure to iodoacetic acid were superimposable only in the zone of antibody excess. In the zones of equivalence the three modified preparations, although similar to each other, differed from untreated samples in the amounts of C′ fixed. After exposure to sonic oscillation, however, the untreated and oxidized highly purified hormone gave fully superimposable C′-fixation curves (Fig. 5).

Discussion

The data presented here have demonstrated that when bovine parathyroid hormone was incubated with iodoacetic acid at *p*H 3.8, methionine was the only amino acid alkylated and that the resulting carboxymethylsulfonium derivative possessed little or no hor-

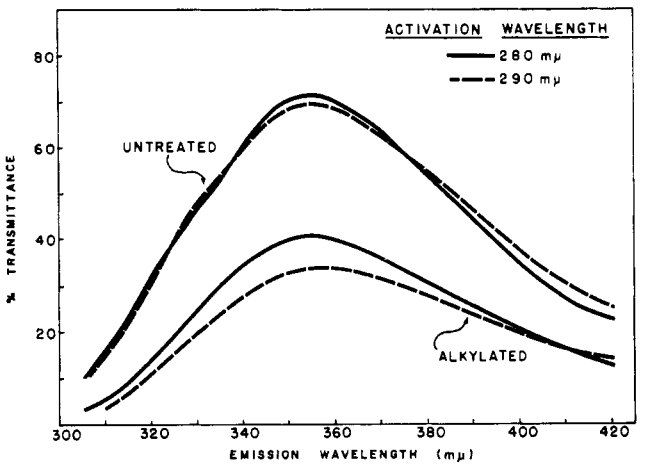

FIG. 4.—Fluorescence-emission spectra of untreated and alkylated highly purified parathyroid hormone in 0.1 N NaOH (the same sample used for measurement of the ultraviolet-absorption spectrum). The uniform decrease in per cent transmittance seen following alkylation reflects dilution of the polypeptide during separation from iodoacetic acid.

TABLE III
AMINO ACID ANALYSES OF SAMPLES OF THE HIGHLY PURIFIED PARATHYROID HORMONE

	Amino Acid Residues per Molecule after Treatment[a]			
	None	Performic Acid Oxidation	Alkylation + Performic Acid Oxidation	H_2O_2 + Alkylation + Performic Acid Oxidation
Lysine	6.8	6.9	6.6	6.9
Histidine	3.0	3.0	3.1	2.9
Ammonia[b]	7.8	10.2	7.9	9.7
Homoserine lactone	0	0	0.05	0
Arginine	3.3	3.5	3.6	3.8
Cysteic acid	0	0.24	0.17	0.29
Methionine sulfoxides	0.19	0	0	0
Aspartic acid	7.5	6.6	7.1	7.1
Methionine sulfone	0	1.02	0.16	0.96
Threonine	1.6	1.3	1.4	1.3
Serine	6.1	4.2	5.7	5.9
Homoserine	0	0	0.16	0
Glutamic acid	8.8	8.2	8.1	9.0
Proline	2.6	3.0	2.3	2.5
S-Carboxymethylhomocysteine	0	0	0.37	0
Glycine	3.9	3.5	3.8	4.0
Alanine	7.0	6.5	6.5	6.6
Cystine	0	0	0	0
Valine	5.5	5.0	5.5	5.6
Methionine	1.08	0.05	0.29	0.05
Isoleucine	2.0	2.0	2.0	2.1
Leucine	7.9	7.8	7.4	7.4
Tyrosine	1.2	0.4	0.05	0.05
Phenylalanine	2.1	2.0	2.1	2.0
Methionine and related residues[c]	1.27	1.07	1.03	1.01

[a] None of the values given has been corrected for decomposition during hydrolysis (6 N HCl, 22 hours, 110°, in sealed glass tubes evacuated by oil pump). [b] Although final purification of the hormone was by gel filtration with an ammonium acetate buffer, the preparation was lyophilized four times from large volumes of freshly boiled deionized water before hydrolysis. [c] Sum of methionine plus methionine sulfoxides, methionine sulfone, homoserine, homoserine lactone, and *S*-carboxymethylhomocysteine.

monal activity *in vivo*. If the reversible inactivation of the hormone by H_2O_2 oxidation also involved methionine alone, prior oxidation should protect against the effect of exposure to iodoacetic acid. This hypothesis was supported by the data in Table II. The activity of oxidized, but not alkylated, hormone could be fully restored by cysteine reduction, and hormone that was oxidized before exposure to iodoacetic acid was also reactivated by subsequent reduction.

These experiments were carried out with a highly purified preparation of bovine parathyroid hormone in which an inactive trace contaminant was detected by starch-gel electrophoresis. In addition, the analytical value for methionine, including methionine sulfoxide, of this preparation before chemical treatment, 1.27 residues per mole, was somewhat less than the mean value of 1.66 given by Rasmussen and Craig (1962a) for comparable preparations. However, Rasmussen and Craig also reported one such preparation with only one methionine residue per molecule, indicating that there is no consistent discrepancy between the two laboratories concerning the methionine composition and suggesting that the true value for the methionine content of the phenol-extracted form of bovine parathyroid hormone is still uncertain. With respect to the rest of the amino acid analysis of the highly purified parathyroid hormone, the values are in reasonable agreement with the previous reports by Rasmussen and Craig (1962a,b). Possibly the apparent discrepancies are not statistically significant, or they may reflect minor heterogeneities in all current preparations of parathyroid hormone. The lower analytical value for methionine in our highly purified parathyroid hormone could also represent the loss, during fractionation and purification, of a small fragment of the molecule that contained a methionine residue unimportant for hormonal activity or it could represent incomplete hydrolysis of a peptide fragment containing methionine.

The results of the amino acid analyses (Table III) indicate directly that methionine was the site of alkylation and oxidation in highly purified parathyroid hormone and that prior exposure to H_2O_2 protected the molecule against alkylation by subsequent incubation with iodoacetic acid. The sum (0.87 residue per molecule) of the hydrolysis products of methionine alkylation (homoserine, homoserine lactone, *S*-carboxymethylhomocysteine, and methionine) plus the methionine sulfone content of the sample (0.16 residue per molecule) was equal to 1.03 residues, as compared with 1.27 residues (methionine, 1.08, plus sulfoxide, 0.19) determined before alkylation (Table III, column 2). The recoveries of lysine and histidine were quantitative, in agreement with the fact that there were no chromatographic peaks in the positions of ε-carboxymethyllysine or 1- or 3-carboxymethylhistidine (Gundlach *et al.*, 1959).

Although it was concluded from the amino acid analyses that both alkylation with iodoacetic acid and oxidation with H_2O_2 at *p*H 3.8 modify only the methionine in parathyroid hormone and that this amino acid is at a site important for hormonal activity, it remained unclear whether it was the chemical transformation of the methionine residue itself or a secondary alteration in structure that rendered the molecule biologically ineffective. Two different methods were used to examine this second possibility.

First, as shown in Figure 1B, oxidation with H_2O_2 did not alter the electrophoretic mobility of the hormone band in the partially purified hormone, indicating that the oxidation had produced no important change in the net charge on the molecule (Smithies, 1959). The lack of a major alteration of electrophoretic mobility offered additional evidence, in agreement with the amino acid analyses, that oxidation did not involve the imidazole ring of histidine or the amino group of lysine, since if they had been affected the mobility would have changed greatly. In experiments not shown here, the electrophoretic mobility of alkylated hormone also was found not to differ significantly or consistently from that of the untreated hormone.

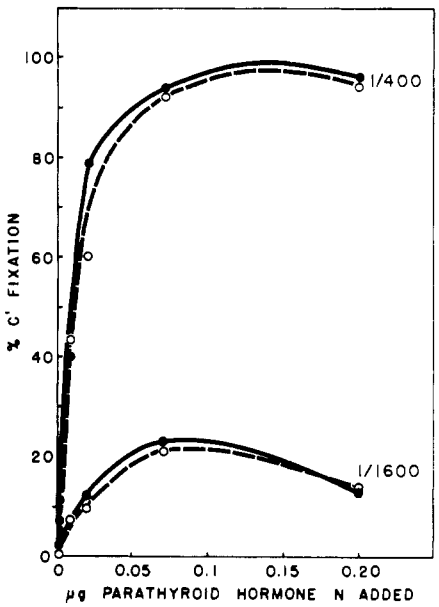

FIG. 5.—C′ fixation between rabbit anti-highly purified parathyroid hormone and the highly purified hormone. The solid lines represent the untreated highly purified hormone and the dashed lines represent the oxidized form. Each point is the mean of duplicate determinations. Both types of preparations were subjected to 3 minutes of sonic oscillation (Tashjian *et al.*, 1964) before addition to the final reaction mixtures. The dilutions of antiserum were 1:400 and 1:1600 for the upper and lower sets of curves, respectively.

Second, the availability of an antibody to the hormone in its reduced, biologically active state (Tashjian, *et al.*, 1962, 1964) offered a method for detecting possible changes in conformation of the molecule after alkylation or oxidation. Correlation of antigenic activity, as measured by C′ fixation, with changes in conformation of the antigen, measured by physical and chemical techniques, has been successfully applied to pepsinogen and pepsin (Van Vunakis *et al.*, 1963; Gerstein *et al.*, 1963), to lactic and triosephosphate dehydrogenases (Kaplan and White, 1963), and to hemoglobin and myoglobin (Reichlin *et al.*, 1963). C′-fixation curves obtained with alkylated and oxidized hormone (both partially purified and highly purified) and with samples first oxidized before exposure to iodoacetic acid were identical. These results were consistent with the amino acid analyses, which indicated that alkylation and oxidation affected the same amino acid. However, changes in C′ fixation in the zones of equivalence, with no change in the zones of antibody excess, for all three modified preparations in comparison with untreated hormone were similar to changes, also seen at equivalence and in antigen excess, in quantitative precipitin reactions in the egg albumin immune system following chemical modification of the antigen (MacPherson and Heidelberger, 1945; MacPherson *et al.*, 1945). The altered precipitin reaction was found to be associated with aggregation of the antigen. Immunochemical studies of parathyroid hormone (partially purified and highly purified) had previously demonstrated that changes in antigenic activity

following freezing and thawing also were probably due to aggregation of the hormone (Tashjian *et al.*, 1964). Therefore it was suspected that the changes in C′ fixation at equivalence following oxidation and alkylation were the result of aggregation. After sonic oscillation, identical C′-fixation curves were obtained with oxidized and unmodified highly purified parathyroid hormone (Fig. 5), indicating that aggregation of the antigen probably was responsible for the altered C′ fixation previously seen at equivalence and that no major conformational change in the molecule followed oxidation or alkylation. Treatment of oxidized or alkylated samples (either partially purified or highly purified parathyroid hormone) with freshly prepared 8 M urea (A. H. Tashjian, Jr., and L. Levine, unpublished data) had the same effect as sonic oscillation in changing the C′-fixation curves for the modified hormones back to a pattern identical with that of untreated hormone. However, the hormonal activity of modified preparations, shown for an oxidized sample in Table IV, was not regenerated following sonic oscillation or exposure to urea, indicating that it was not aggregation *per se* that rendered the molecule hormonally ineffective. As shown in Table IV, the biological activity of untreated highly purified parathyroid hormone was not affected by sonic oscillation or exposure to 8 M urea.

TABLE IV
EFFECTS OF SONIC OSCILLATION AND 8 M UREA ON THE HORMONAL ACTIVITY OF UNTREATED AND OXIDIZED HIGHLY PURIFIED PARATHYROID HORMONE

Solution	No. of Rats	Plasma Calcium[a] (mg/100 ml)
Highly purified parathyroid hormone[b]	5	9.6 ± 0.46
Highly purified parathyroid hormone + sonic oscillation	5	9.7 ± 0.46
Highly purified parathyroid hormone + 8 M urea	5	10.2 ± 0.46
Oxidized highly purified parathyroid hormone[c]	4	7.6 ± 0.51
Oxidized highly purified parathyroid hormone + sonic oscillation	5	7.0 ± 0.46
Oxidized highly purified parathyroid hormone + 8 M urea	5	7.0 ± 0.46

[a] Mean ± standard error. [b] Highly purified parathyroid hormone, 20 μg per rat in all groups. [c] There were no statistical differences between oxidized highly purified parathyroid hormone, oxidized highly purified parathyroid hormone + sonic oscillation, and oxidized highly purified parathyroid hormone + 8 M urea, nor between the three unoxidized samples. The difference between oxidized versus unoxidized samples, however, was highly significant ($P < 0.001$).

As no change in either charge or conformation in the hormone has been shown by starch-gel electrophoresis or by immunochemical techniques, the reason why alteration of methionine renders the molecule hormonally inactive remains unclear. As with ribonuclease (Vithayathil and Richards, 1960), the possibility that methionine is involved in the binding of the hormone to a specific receptor site in target tissues should be considered.

Since the hormonally inactive, oxidized preparation after sonic oscillation was serologically indistinguishable from the unmodified, hormonally active sample (Fig. 5), it must be concluded that the antigenic activity of the hormone does not depend on intact methionine residues important for hormonal activity. Despite the dissociation of hormonal activity from antigenicity, this same anti-highly purified parathyroid hormone has been used to develop an immunoassay method for parathyroid hormone that gives results which agree well with those derived from biological assay of the same samples over a wide range of specific activities (Tashjian *et al.*, 1964). It must follow, then, that most of the hormone samples assayed did not contain large amounts of altered methionine or substantial overestimation of potency would have occurred in the immunoassays. Whether this will interfere with the use of immunoassay methods to estimate physiologically active parathyroid hormone concentrations *in vivo* is uncertain. At least there are no data to suggest that the mechanism of hormonal inactivation *in vivo* is by oxidation or alkylation of methionine.

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