Further Studies on Acetamidination as a Technique for Preparation of a Biologically Valid $^3$H-Labeled Tracer for Parathyroid Hormone

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SUMMARY

The technique of acetamidination of amino groups in parathyroid hormone (PTH) for the purpose of preparation of a tritiated, biologically valid tracer for this hormone, has been more extensively studied. It was found that eight of the ten amino groups in PTH are readily reactive but that two appear unreactive. The kinetics of labeling suggest that preparations of PTH which are labeled to this level of 80% of theoretical consist of a homogeneous population of molecules in which each PTH molecule contains eight tritiated acetamidino groups. There is no question of the presence of unlabeled hormone in such preparations.

Eighty per cent labeled acetamidino-PTH is identical, quantitatively and qualitatively, in its biological activity with native PTH as shown by three accepted bioassays: serum calcium elevation, urine phosphate excretion, and activation of kidney cortex plasma membrane adenylate cyclase in vitro. The dose-response relationships are identical for labeled and native hormone in all three systems.

Tritiated acetamidino-PTH tends to lose biological activity on storage but full activity can be regained by reduction of the hormone with excess cysteine. However, cysteine reduction conducted at 80° for 2 hours causes some loss of tritium from the hormone. Therefore, dithiothreitol reduction at room temperature was utilized to maintain biological activity.

Eighty per cent acetamidino-PTH can be further purified by ion exchange chromatography on carboxymethylcellulose using a continuous gradient of sodium acetate in 8 M urea. Such chromatography reveals the presence of isohormones in both cold and radioactive PTH. Co-chromatography of tritiated acetamidino-PTH with a cold isohormone of PTH shows that while the isohormones can be separated from one another, the acetamidino derivatives of each isohormone elute in a virtually identical position with their parent unlabeled PTH molecule.

In 1972, we reported the preparation of a biologically active derivative of parathyroid hormone labeled with high specific activity tritium (1, 2). This derivative, acetamidino-PTH, was active in the in vitro adenylate cyclase assay system described by Marcus and Aurbach (3), and in elevating the serum calcium of thyroparathyroidectomized rats. It was also successfully utilized in establishing the existence of specific binding of PTH to kidney cortex plasma membranes in vitro (4). The radioactive hormone was readily prepared under mild conditions, with generation of no detectable side products. The labeling procedure was also utilized to prepare tritiated insulin, and the acetamidino derivatives of insulin were extensively characterized (5).

However, in this previous work we utilized acetamidino PTH preparations of a variable extent of labeling, some preparations containing as much as an average of seven labeled amino groups per hormone molecule, and some with as little as one to two labeled groups. We also observed a considerable variability in the biological potencies of the tritiated hormone preparations although qualitatively the labeled hormone appeared very similar to native PTH.

Therefore, we have undertaken a more extensive investigation of the labeling of PTH with acetimidate, and of the biological and chemical characteristics of the labeled hormone. The data presented here strongly support our earlier conclusions that with the proper precautions in its preparation and handling, acetamidino-PTH is a fully valid and useful tracer for native parathyroid hormone.

EXPERIMENTAL PROCEDURES

Parathyroid Hormone—PTH was purchased from the Wilson Co., Chicago. This material was then subjected to further purification, nitrogen analysis, and biological activity analysis as described below.

[3H]Acetonitrile—High specific activity [3H]acetonitrile was custom synthesized by New England Nuclear Co. Specific activities obtained ranged from 1.2 Ci per mmol in earlier preparations to 2.2 Ci per mmol in later preparations; the material was purchased in 1-Ci lots. It was converted to methyl[3H]acetimidate by reaction with equimolar amounts of dry HCl gas and methanol, as described earlier (1, 5).

Hormone Labeling—Reaction of the crystalline methyl[3H]acetimidate with PTH was conducted basically as described earlier.
One to two milligrams of [3H]imidate was incubated for 2 hours at 0°C in a N₂ atmosphere with 100 to 500 μg of PTH at pH 9.0, in N₂-saturated 0.025 M borate buffer. The reaction volume was 100 to 200 μl. The labeled hormone was then separated from the reaction mixture as described below.

All animals used for PTH bioassay were kept on this diet for no less than 4 days and no longer than 10 days prior to use.

In Vivo Bioassays—Rats were surgically thyroparathyroidectomized under ether anesthesia. After recovery from the anesthetic (10 to 15 min), intraperitoneal injection of hormone or carrier solutions (1 to 10 mM acetic acid) were given in 0.1 to 0.3 ml volume. A second injection of hormone or carrier was given 2% hours after the first. During the 5-hour period following surgery, urine was collected, and at the end of this period, the animals were killed by decapitation and the blood was collected for serum calcium analysis. Serum calcium was determined colorimetrically using the calcium-stat kit purchased from Pierce Chemical Company, Rockford, Ill. Urine phosphate was determined by the Fiske and SubbaRow technique as described for urine by Allport and Keyser (6).

Chromatography—Experimental details for the individual chromatographic systems are given below. All columns were run in 8 M urea unless otherwise indicated.

Hormone Concentrations—Unless otherwise indicated, all hormone concentrations are based on analysis of total nitrogen by the indanetrione hydrate procedure. Samples were sealed in glass tubes and digested in concentrated H₂SO₄ at 480° for 90 min. Aliquots were then analyzed as described by Jacobs (7).

RESULTS

All of the experiments reported here were done with commercially prepared PTH.² PTH prepared by gradient elution from CMC has been shown to be homogenous in a variety of systems, although small amounts of isohormones have recently been detected by Keutman et al. (8). We directly investigated the purity and the biological activity of the Wilson "highly purified" material which is prepared by CMC chromatography. Fig. 1 shows the results of a gel electrophoresis experiment of the hormone run in 15% acrylamide-8 M urea as described by Hawker et al. (9), illustrating the presence of a single band of peptide material in this system. This result, of course, does not unequivocally rule out the presence of trace contaminants, or of isohormones, which indeed are present as is demonstrated later in this paper. Nonetheless, it appeared that the dominant material in this commercial preparation was a peptide very similar to PTH in its electrophoretic behavior.

On the other hand, we found that the standard, 1-mg lots of this "highly purified" material appeared to be highly contaminated with salt since analysis of total nitrogen by the indanetrione hydrate method always yielded an actual protein content varying from 500 to 700 μg per mg of total material, based on 18.4% nitrogen in PTH. We established that the nitrogen present in these preparations was protein and not ammonium or other nitrogen salts by precipitation of the hormone with trichloroacetic acid prior to the nitrogen analysis. This treatment always totally removed nitrogen from the sample.

The biological activity of the highly purified hormone was assayed on a microgram of protein basis using thyroparathyroidectomized rats. The results of these assays are shown in Fig. 2.

The Wilson Company currently makes four types of PTH: (a) parathyroid substance, defatted glands; (b) trichloroacetic acid powder; (c) purified PTH prepared from trichloroacetic acid powder by gel filtration on Sephadex; and (d) highly purified PTH prepared from e by gradient CMC chromatography in absence of urea.

FIG. 1. Disc electrophoresis gel of highly purified Wilson PTH run in 15% acrylamide-8 M urea, pH 4.3. Electrophoresis was conducted for 3 hours at 4°, 3 ma per gel. Sample size, 30 μg.

FIG. 2. Dose-response curves for highly purified Wilson PTH in thyroparathyroidectomized rats. The bioassay technique is described in the text.

The hormone is very active in elevating serum calcium, and a full return to normal calcium levels (10 mg/100) in thyroparathyroidectomized rats can be achieved in 5 hours with injection of 8 to 10 μg of this material under the conditions of our bioassay.

From these results it appeared that the highly purified hormone would be satisfactory for further studies of the amidinating reaction and the properties of the labeled hormone. We therefore proceeded to investigate more fully the maximum extent of labeling of hormone which could be achieved, and the biological activity of such extensively labeled hormone. Earlier we had noted that up to 70% of the theoretical value for labeling PTH (7 out of 10 amino groups) could be achieved with retention of biological activity (1, 2), so it appeared possible that under appropriate conditions, complete modification might be possible.

Highly purified Wilson PTH was incubated with a 10 molar excess of methyl[3H] acetimidate for varying periods of time up to 2 hours. The reaction was stopped by passage of the incubation mixture through a column of Bio-Gel P-2 which results in nearly total separation of the labeled hormone from the reaction mixture in about 10 min. Fig. 3 shows the excellent separation of the hormone from the smaller radioactive molecules (imidate and hydrolyzed imitate) by this column and describes the conditions for optimal use of this P-2 column for this purpose. We found...
that it was essential to use the large size Bio-Gel beads (50 to 100 mesh), since the 200 to 400 mesh Bio-Gel P-2 showed a significant retardation of the hormone, probably due to nonspecific binding. Use of 1.0 mM acetic acid rather than 50 mM also resulted in significant retardation of the hormone and poorer separation. In addition, it was found that column age was critical and that a significant retardation of the hormone and poorer separation occurred. This result was further explored by attempting to label more extensively an isolated sample of 80% amidinated PTH by addition of a second large molar excess of imidate. After a 2-hour reaction period, the labeled hormone was again passed through Bio-Gel P-2 and its specific radioactivity was determined. As indicated by the triangles in Fig. 4, this treatment produced no further labeling of the hormone, since the specific activity is identical with that of the initial material. Thus, 80% of theoretial, that is, a total of eight amino groups per parathyroid hormone molecule, appear to represent the maximum extent to which the hormone can be labeled with the imidate reagent under the conditions utilized.

The biological activity of 80% labeled PTH was investigated initially by comparison with cold hormone and with an active 50% labeled PTH sample, prepared at an earlier date, in a single dose assay. As shown in Table I it became clear that both labeled hormone preparations were less active than the native hormone, and in particular that the older tritiated hormone which was active at an earlier date had lost considerable biological activity. Since secondary radiation damage frequently can occur in highly radioactive chemicals due to generation of free radicals (particularly oxygen radicals in aqueous solution), and since PTH is particularly sensitive to oxidation of methionine by oxygen radicals (H₂O₂), we investigated whether a reversible oxidation of methionine might have occurred in the tritiated hormone preparations. Separate samples of active hormone were oxidized by H₂O₂, and reduced by heating with excess cysteine as described by Tashjian and Munson (10). Eighty percent labeled [³H]PTH was treated similarly, as was a sample of “old” labeled hormone, and all samples were bioassayed. Table I also shows the data from all of these single dose bioassays. It is clear from these data that the activity of the 80%-labeled PTH and the “old” [³H]PTH was elevated to that of the native hormone by reduction with cysteine. Oxidation of all of the samples eliminated biological activity. The data, therefore, support the belief that oxidation of methionine catalyzed by radiation-generated free radicals may occur to some extent during extensive

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**Table I**

| Hormone preparation | Serum calcium from bioassay |
|---------------------|-----------------------------|
|                     | Original | Oxidized | Reduced |
| “Old” PTH (50%-labeled) | 7.9 ± 0.8 | 6.8 ± 0.5 | 11.0 ± 0.3 |
| 80% amidinated PTH   | 12.2 ± 0.5 | 7.2 ± 0.8 | 12.0 ± 0.7 |
| Native PTH           | 12.5 ± 1.0 | 7.2 ± 0.2 | 12.1 ± 1.1 |
labeling and during storage of labeled hormone. Therefore, it is important to reduce fully such hormone preparations prior to biological experiments, and to maintain the hormone in a reducing environment.

A dose-response curve for fully reduced 80% labeled PTH was next obtained by measurement of both serum calcium and urine phosphate in thyroparathyroidectomized rats. Fig. 5 shows that within the error of this assay the [3H]PTH is identical with the control native hormone preparation in its ability to elevate serum calcium. For these experiments a control group of animals was run on the same day as each experimental group so that the data are presented as an increase in serum Ca²⁺ over those thyroparathyroidectomized controls. Fig. 6 indicates that excretion of phosphate in the urine is also dramatically elevated by the [3H]PTH compared to the native hormone. Thus, these data appear to provide unequivocal evidence that 80% acetamidino-PTH is virtually identical with the native hormone in its ability to elevate serum calcium and urine phosphate, the two most characteristic activities of parathyroid hormone.

A point of some concern to us, however, related to the conditions required for reduction of the hormone. As described above, this procedure requires heating the hormone to 80°C in 0.1 M cysteine at acid pH for 2 hours. It seems possible that under these conditions some ¹²¹I exchange might occur, or perhaps some hydrolysis of the hormone to smaller fragments, or hydrolysis of the PTH imidate residue itself. PTH is known to be sensitive to mild acid hydrolysis at the Gln₅₈-Asp₇₀ peptide bond (10) and such a chemical change might be undetected in the bioassay since the 1-29 fragment of PTH thus produced has biological activity (10). As shown in Fig. 7, gel filtration of the 80% acetamidino-PTH on a Bio-Gel P-10, 8 M urea column did indeed suggest that some degradation of the hormone had occurred. The main peak elutes in a position consistent with PTH, but two smaller fragments were also observed, suggesting that some degradation of the hormone may have occurred. An alternative explanation for the appearance of these secondary radioactive peaks was the possibility that trace contaminants existed in the original hormone preparation. Such contaminants might be undetected in gel filtration of the original hormone, but might be highly reactive with the imidate, thereby generating detectable peaks upon labeling.

Because of the above uncertainties, we added two modifications.
TABLE II

Biological assay of dithiothreitol-reduced 80% labeled [%H]PTH compared to cysteine-reduced [%H]PTH

| Hormone injected | Serum calciuma | Urine phosphatel |
|------------------|----------------|-----------------|
| None             | 7.80 ± 0.7     | 2.00 ± 0.4      |
| 10 pg of DTT-reduced | 10.41 ± 1.1   | 7.73 (3.0-20.0)c |
| 10 pg of cysteine-reduced | 9.80 ± 1.1   | 10.10 (5.0-18.0)c |

a Average in milligrams per 100 of five rats 5 hours after thyroparathyroidectomy ± S.D.
b Total milligrams of phosphate excreted in 5 hours after thyroparathyroidectomy.
c The variability is shown as a range, rather than ± S.D. because of the extreme variation in the urine phosphate.

Fig. 8. Purification of purified Wilson PTH on carboxymethylcellulose. Ten milligrams of PTH dissolved in 30 ml of 0.01 M sodium acetate-8 M urea-1 mM DTT, pH 4.9, were applied to a column (0.5 x 6 cm) equilibrated in the same solution. After the hormone had entered the column, a gradient of 0.01 to 0.2 M (pH 6.0) sodium acetate (8 M urea-1 mM DTT, 70 ml in each reservoir) was begun (---). After elution of the two hormone peaks, the remaining material present in the column was eluted with 1 M NH₄ acetate (pH 6.9). Fraction size is 0.5 ml.

to our procedures for producing [%H]PTH. First, the use of dithiothreitol as a reducing agent in place of cysteine was investigated. As indicated in Table II, 80% [%H]acetamidino-PTH kept in 50 mM DTT does indeed retain apparent full biological activity, and we therefore turned to this agent to maintain the hormone in the fully reduced state.

Second, in order to eliminate any question as to the homogeneity of the original hormone we initiated an effort to purify extensively PTH from the less homogenous commercially available Wilson material, by chromatography on CMC in 8 M urea as described by Keutman et al. (8). For this work we purchased

10 mg of "purified" PTH and chromatographed it directly on a small CMC column prepared in a Pasteur pipette (0.4 x 6 cm). In order to avoid contamination of the hormone with ammonium ion so that specific activities could be based directly on nitrogen analysis, we utilized sodium acetate in place of ammonium acetate in the gradient. Fig. 8 shows the chromatographic profile obtained by this procedure. Large amounts of ultraviolet absorbing material did not stick to the column and this material was washed completely out of the column before starting the gradient, as indicated. Initiation of the gradient resulted in elution of a broad band of ultraviolet absorbing material, spread over the entire gradient, several minor peaks, and two major components (I and II). The remaining ultraviolet absorbing material was then eluted with 1 M ammonium acetate. The broad base-line of ultraviolet absorption is believed to result from the oxidation of sulfhydryl groups of DTT to disulfide as reported earlier by Keutman (8). This interpretation is supported by the observation that the base-line drops back to its original value when the column was washed with 1 M ammonium acetate which contains no DTT.

Of the two principal peaks eluted by the gradient, Peak II elutes in a position consistent with that expected for the dominant form of PTH reported by Keutman et al. and Peak I elutes in a position compatible with a major isoform described by Keutman. This latter material is present in quite significant amounts in this preparation. It should also be noted that we did not observe the third isof orm reported by Keutman et al. which elutes after the dominant form of the hormone. However, small amounts of this isof orm may be present in some of the tritiated hormone preparations prepared earlier from highly purified PTH (e.g. see Fig. 11).

Peaks I and II were rechromatographed on the sodium acetate-urea gradient and each eluted as a single symmetrical peak of ultraviolet absorbing material (Fig. 9). Both materials were then bioassayed in the rat to confirm that these peaks did indeed
### Table III

| Hormone injected | Serum calcium | Urine phosphate |
|------------------|---------------|-----------------|
| None             | 7.7 ± 0.5     | 1.8 ± 0.9       |
| 6 μg of Peak II  | 9.1 ± 0.9     | 4.8 (3-14)      |
| 12 μg of Peak II | 9.9 ± 0.5     | 9.0 (7-11)      |
| 7 μg of Peak I   | 10.4 ± 0.5    | 9.0 (4-15)      |
| 14 μg of Peak I  | 11.4 ± 1.1    | 10.3 (6-14)     |

* Average in milligrams per 100 of five rats 5 hours after thyro-parathyroidectomy ± S.D.

* Total milligrams of phosphate excreted in 5 hours after thyro-parathyroidectomy.

* The variability is shown as a range, rather than ± S.D. because of the extreme variation in the urine phosphate.

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**DISCUSSION**

This paper confirms and considerably extends our earlier report that acetamidino-PTH is a biologically active derivative of the
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is due to unlabeled PTH in the preparations. Furthermore, the amidinating reaction produces a valid and highly useful hormone, and that introduction of tritium into the hormone by dino-PTH and cold Peak I material from Fig. 8. Conditions were as described for Fig. 8.

As indicated above, it is critical to have a fully reduced PTH derivative in order to retain biological activity. We have been able to substitute 50 mM DTT at room temperature for 100 mM cysteine at 80° to reduce the hormone so that any possible hormone degradation due to reduction is eliminated. As we reported earlier (4), the inverse correlation between membrane binding of PTH to kidney receptor in vivo and oxidation of hormone by H₂O₂ is an interesting one and probably explains the loss of biological activity in oxidized PTH. It now seems likely that a direct measure of the extent of oxidation of PTH in future hormone preparations should be obtainable by a standardized membrane-binding assay.

In our earlier report (2) we showed a dose-response relationship between PTH and activation of rat kidney cortex adenylate cyclase identical with that reported for PTH in other laboratories (3, 11). The present paper shows similar bioassays for elevation of serum calcium which is the classic bioassay for PTH, and for elevation of urinary phosphate. Thus, the amidaminodino-PTH derivative has now been demonstrated to be quantitatively as active as native hormone in both of the accepted quantitative bioassay systems, and additionally its effect on urine phosphate elevation is identical with that of the native hormone. Thus, there is a firm basis for concluding that the tritiated derivative is a fully valid tracer for PTH. In further support of this conclusion, it should be noted that we have demonstrated binding of [3H]PTH to isolated kidney plasma membranes in vitro (4) which is specific for only active PTH.

The concept of the general utility of acetimidination as a technique for labeling peptides containing free amino groups is considerably strengthened by the data presented in this paper.
The acetamidino-lysine residue is clearly very similar to the lysine free amino group and in many cases where a lysine residue is critical to biological activity it may nonetheless be possible to acetamidinate without loss of such activity. In fact, acetamidino lysine is sterically quite similar to arginine, and it is well known that the conservative replacement of arginine for lysine frequently does not detectably modify biological function in proteins. In fact, in one of the earliest studies of amidination and its effects on biological function, Wofsey and Singer (14) found that binding of antigens to certain rabbit antibodies was not altered in any way by exhaustive amidination of antibody amino groups. In practice, we have found that both PTH and insulin can be extensively acetamidinated without detectable alteration in biological activity (1, 2, 5), and we have likewise found that ACTH can be acetamidinated and still retain biological potency.  

Acknowledgment—We are grateful to Mrs. Heather Petrolla for her expert technical assistance.

T. Saito, J. Kowal, and J. E. Zull, unpublished

REFERENCES
1. Zull, J. E., and Repke, D. W. (1972) J. Biol. Chem. 247, 2183
2. Zull, J. E., and Repke, D. W. (1972) J. Biol. Chem. 247, 2195
3. Marcus, R., and Aurbach, A. D. (1969) Endocrinology 86, 801
4. Malbon, C. H., and Zull, J. E. (1974) Biochem. Biophys. Res. Commun. 56, 952
5. Repke, D. W., and Zull, J. E. (1972) J. Biol. Chem. 247, 2189
6. Allport, N. L., and Keyser, J. W. (1957) Colorimetric Analysis 2nd Ed Vol. 1 pp. 277-279, Chapman and Hall, London
7. Jacobs, S. (1959) Nature 183, 262: (1960) Anal. Biochem. 85, 257
8. Keutman, H. T., Aurbach, G. D., Dawson, B. F., Niall, H. D., Defos, L. J., and Potts, J. T. Jr. (1971) Biochemistry 10, 2779
9. Hawker, C. D., Glass, J. D., and Rasmussen, H. (1966) Biochemistry 5, 344
10. Keutman, H. T., Dawson, B. F., Aurbach, G. D., and Potts, J. T. Jr. (1972) Biochemistry 11, 1973
11. Dousa, T., and Rychlík, L. (1968) Biochim. Biophys. Acta 158, 484
12. Tregear, G. W., Van Rietschoten, J., Greene, E., Keutman, H. T., Niall, H. D., Reit, B., Parsons, J. A., and Potts, J. T. (1973) Endocrinology 93, 1349
13. Potts, J. T. Jr., Tregear, G. W., Keutman, H. T., Niall, H. D., Sauvé, R., Defos, L. J., O’Riordan, J. L. H., and Aurbach, G. D. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 63
14. Wofsey, L., and Singer, S. J. (1963) Biochemistry 2, 104
Further studies on acetamidination as a technique for preparation of a biologically valid 3-H-labeled tracer for parathyroid hormone.

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*J. Biol. Chem.* 1975, 250:1668-1675.

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