Studies on the Biosynthesis in Vitro of Parathyroid Hormone

II. THE EFFECT OF CALCIUM AND MAGNESIUM ON SYNTHESIS OF PARATHYROID HORMONE ISOLATED FROM BOVINE PARATHYROID TISSUE AND INCUBATION MEDIUM*

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SUMMARY

Bovine parathyroid gland slices were incubated in vitro in buffers containing concentrations of calcium in the range of 0.5 mM to 2.0 mM and magnesium in the range of 0.2 mM to 1.7 mM and either a 3H- or a 14C-amino acid. After incubation, radioactive parathyroid hormone was isolated from both the tissue and the incubation medium by a combination of methods including gel filtration and ion exchange chromatography. The isolation, localization, and quantification of parathyroid hormone present at various stages of purification was assisted by adding to the samples, prior to processing, small amounts of biologically synthesized, highly purified radioactive parathyroid hormone as an internal standard. The use of the internal standard to correct for losses of parathyroid hormone during processing was particularly necessary due to the finding that a large fraction of radioactive parathyroid hormone which was previously biosynthesized and which was employed as an internal standard and marker could not be found in the incubated tissue, we could not find any in the incubation medium. Whereas hormonal activity was readily measured in extracts of the incubated tissue, we could not find any in the incubation medium. The use of the internal standard was necessary to ensure that the radioactive hormone eluting from Sephadex columns in the region where parathyroid hormone elutes. Neither group, however, provided data to indicate whether or not changes in ionic concentration affected incorporation of amino acid into the isolated hormone, itself.

The present report expands on our earlier work (4) and includes data on the isolation of newly synthesized hormone from both tissue and incubation medium and the effect of both calcium and magnesium on synthesis. Quantitation of the amount of parathyroid hormones in samples, incorporation of radioactivity, and the localization of parathyroid hormone during isolation have been achieved through the use of purified radioactive parathyroid hormone which was previously biosynthesized and which was employed as an internal standard and marker.

METHODS

Incubation of Tissue Slices and Preparation of 14C- or 3H-Parathyroid Hormone-containing Trichloroacetate Powder—Parathyroid gland slices were prepared and incubated as described previously (4) in Krebs-Ringer phosphate buffer, pH 7.4, containing a concentration of calcium specified below and either radioactive leucine or lysine. The concentration of magnesium was 0.83 mM unless otherwise specified. For preparation of batches of radioactive parathyroid hormone to be used as an internal standard, 15 to 20 g of slices were incubated in 100 ml of buffer with a calcium concentration of 0.5 mM and either 1

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µCi per ml of 14C-amino acid (leucine or lysine) or 5 µCi per ml of 3H-amino acid (leucine or lysine) (Schwarz BioResearch). After incubation the tissue and medium were separated. The slices were rinsed and frozen until they could be processed to the trichloroacetate powder stage as described earlier (4). The yield of trichloroacetate powder was about 1 mg per g for amounts of tissue in the 1- to 2-µg range and about twice that for the 15- to 20-µg batches of slices. The trichloroacetate powders were processed first on a Sephadex G-100 column and then on a Cm-cellulose column as described below. Incubation media were clarified by centrifugation, lyophilized when necessary to reduce volume, and processed by column chromatography (below).

Column Chromatography—Columns (2.5 × 45 cm) were packed with Sephadex G-100 superfine (Pharmacia) and equilibrated either with 0.14 M ammonium acetate-0.007 M mercaptoethanol buffer, pH 4.8 (System A), for processing trichloroacetate powders derived from tissue or with 0.01 ammonium acetate buffer, pH 6.8 (System B), for processing incubation media. Samples were applied in a small volume of the buffer used to equilibrate the column. Fractions were usually 2.5 ml. Flow rates were 8 to 15 ml per hour.

Cm-cellulose columns (1 × 10 cm) were prepared using CM-52 (Whatman) in a slurry in 0.01 M ammonium acetate-0.007 M mercaptoethanol buffer, pH 5.3. Samples were applied in the same buffer but after which a linear conductivity gradient was developed from 0.01 M, pH 5.3, to 0.33 M ammonium acetate, pH 7.0, over a volume of 1200 ml. Fractions (6.0 ml) were collected at flow rates of 30 to 35 ml per hour. Conductivity and pH were monitored over the course of the elution to ensure that proper development of gradient was attained. When specified in the text, 8 µl urea was included in the gradient according to the methods of Potts and the chromatography was conducted at room temperature to prevent precipitation of the urea.

Gel Electrophoresis—Analytical gel electrophoresis was performed using standard Canaletto equipment. Gels containing either 10% or 15% acrylamide were cast in glass tubes with 0.5% bisacrylamide. Ten per cent gels were run at pH 5.0 in 0.33 M β-alanine in 0.2 M acetic acid solution for 140 min at 3 ma per gel. Fifteen per cent gels were run in the same solution at pH 4.08 for 100 min or in 0.35 M glycine in 0.25 M acetic acid at pH 2.75 for 60 min, both at 4.1 ma per gel. The gels were then fixed and stained in Coomassie blue for 60 min.

Bioassays of Parathyroid Hormone—The assay system of Munson (7) was used with slight modification. The rats (female, A. R. Schmidt strain) were placed on a low calcium diet 3 days before the assay. Parathyroidec tom needs was performed by electrolytrea cuture. Serum calcium concentration was determined by a complexometric method (4) in samples obtained 4 hours after injection of standard preparations or unknowns.

Radioimmunoassay Guinea pig antiserum against partially purified bovine parathyroid hormone was used. Parathyroid hormone was prepared in our laboratory using conventional procedures (8) and was iodinated with 125I (Cambridge Nuclear Corporation, Cambridge, Massachusetts), according to the method of Hunter and Greenwood (9). The radioimmunoassay was performed using the technique of Schopman (10). Standard curves B/F (ratio of radioactive hormone bound to antibody to free hormone) versus amount of nonradioactive parathyroid hormone were obtained for the range of 30 to 3000 pg. Both the whole incubation medium and the biosynthesized parathyroid hormone isolated from the tissue slices were tested at multiple dilutions in order to compare the shape of the curves for the unknown samples with that of the standard hormone preparation. A variety of changes in ionic composition of media and vehicle used for dissolving parathyroid hormone were also tested. These were not found to have any effect on the B/F ratios.

Measurement of Radioactivity Aliquots of column eluates were added to Bray's solution (11) for determination of radioactivity. When gel slices were to be counted they were first dissolved in H2O2 as described earlier (4). All assays were performed with a Packard liquid scintillation counter model 30675 and were corrected to absolute disintegrations with the use of internal standards.

Amino Acid Analysis—Amino acid analysis was performed after a 40-hour hydrolysis of the sample in a vacuum in triple distilled 6 N HCl at 110°C. The analysis was performed on a Beckman model 120 analyzer equipped with 20-mm flow cells, using the accelerated flow rate procedure.

Other Analyses, Assays, and Sources of Chemicals—Protein analysis was performed by the method of Lowry et al. (12), using bovine serum albumin as standard. Absorbance of column eluates was determined in a Gilford model 1200 spectrophotometer. Partially purified parathyroid hormone at a potency of 150 units per mg of protein was purchased from Wilson Laboratory and is designated in the text as trichloroacetate powder (Wilson). All other methods were described previously (4).

RESULTS

Preparation and Purification of 3H- and 14C-Parathyroid Hormone

For the preparation of radioactive parathyroid hormone, milligram quantities of either 14C- or 3H-trichloroacetate powder prepared as described under "Methods" were purified sequentially on Sephadex G-100 and Cm-cellulose. Elution profiles typified by the one shown in Fig. 1A were obtained following Sephadex chromatography. The protein peak identified by the arrow was predominantly parathyroid hormone as verified by bioassay and gel electrophoresis. The over-all pattern of absorbance differed from that reported previously (4) in that a major protein component eluting in the region of the void volume (Fraction 20) was much reduced. The difference is attributable to the mixing in the earlier studies of our trichloroacetate powder samples with commercial trichloroacetate powder (Wilson). The latter contained large amounts of the early eluting component whereas the trichloroacetate powder prepared by us does not. The finer resolution of the peaks of protein and radioactivity shown here compared to that obtained earlier is due to the use of G-100 superfine rather than the standard grade. Fig. 1A shows that there was one major peak of radioactivity, the maximum of which preceded the maximum absorbance of the hormone peak (Fractions 50 to 57) by a few milliliters. This lack of correspondence between radioactivity and absorbance was characteristic of this step of purification.

When the contents of the tubes through the peak of radioactivity were pooled and chromatographed on Cm-cellulose,
Studies on Biosynthesis in Vitro of Parathyroid Hormone. II

Vol. 246, No. 10

Fig. 1. Gel filtration and ion exchange chromatography of radioactive trichloroacetate powder prepared from tissue. A, 24 mg of 14C-trichloroacetate powder protein containing $2.4 \times 10^4$ dpm (1-hour incubation of slices) were applied and eluted as described under “Methods.” Aliquots of 0.5 ml each were sampled for assay of radioactivity. Fractions 45 to 57 contained a total of $1.4 \times 10^4$ dpm and 6.7 mg of protein. B, 2.8 mg of lyophilized powder containing 583,000 dpm resulting from A, above, were dissolved in 5.0 ml of starting buffer and applied to a Cm-cellulose column. The elution schedule was as described under “Methods.” Fractions of 6.0 ml were collected. Aliquots (0.2 ml) were sampled for assay of radioactivity. Fractions 48 to 56 contained the hormonal activity. 14CP parathyroid hormone (700 µg) containing $2 \times 10^4$ dpm was obtained. The pattern typified by the one shown in Fig. 1B was obtained. About 60% of the radioactivity applied to the Cm-cellulose column was eluted by the standard elution conditions (8). Of the amount recovered, the hormone (Fractions 48 to 56) usually contained more than 50% of the radioactivity, with the bulk of the remainder eluting primarily as a single late emerging band (Fractions 92 to 100). Thus, the radioactive hormone constituted about ¼ of the total radioactivity in the major radioactive peak in the Sephadex profile (Fig. 1A). This was verified in other experiments by separating the major Sephadex radioactivity peak into front and rear halves and chromatographing each separately on Cm-cellulose. When this was done, it was found that the majority of the radioactivity in the front half of the Sephadex peak emerged from the column as a single band centered at an elution volume of about 600 ml (equivalent to Fractions 92 to 100), while the radioactivity in the rear half of the Sephadex peak emerged as a single band centered at a volume of about 300 ml (equivalent to Fractions 48 to 56) coincident with authentic parathyroid hormone. At present, the identity of the material eluting from Cm-cellulose at a volume of 600 ml is not known, but is the subject of continuing investigation. For final preparation of radioactive parathyroid hormone, the hormone was usually rechromatographed on Cm-cellulose. This procedure yielded radioactive parathyroid hormone with specific activities of up to $3000$ dpm per µg for 3H-parathyroid hormone and $700$ dpm per µg for 14C-parathyroid hormone, dependent upon the incubation period (1 to 4 hours); the longer periods yielded the higher specific activities.

As an additional test of purity, preparations of the radioactive hormone were rechromatographed on Cm-cellulose using an ammonium acetate gradient which included 8 M urea according to the methods of Potts. The hormone emerged under these conditions as a single band at a conductance of about 4 mmho and a pH of about 6 (T = 25°C).

Analysis of the purified radioactive parathyroid hormone by analytical gel electrophoresis at three different pH values and two gel concentrations indicated that the parathyroid hormone was more than 90% pure. Some representative gels are shown in Fig. 2. When gels such as these were cut into 2-mm sections (including sample and stacking gels) and the dissolved gel solutions measured for radioactivity, more than 90% of the applied radioactivity was found in the major protein band, an additional 2 to 3% was found in the region of the interface between the stacking and separating gels, and the remainder was unaccounted for. When tested by radioimmunoassay, the biosynthesized hormone reacted in an identical fashion to the standard hormone throughout the entire assay range.

Table I lists the amino acid composition of the purified radioactive parathyroid hormone. The minimum molecular weight calculated from this composition is 9800. These data are in excellent agreement with the recent results of Brewer and Ronan.
Amino acid composition of radioactive parathyroid hormone

| Amino acid | Residues per mole |
|------------|-------------------|
| Lysine     | 9.1               |
| Histidine  | 4.2               |
| Arginine   | 5.5               |
| Aspartic acid | 8.8        |
| Threonine  | 0.1               |
| Serine     | 0.9               |
| Glutamic acid | 11.0        |
| Proline    | 2.1               |
| Glycine    | 4.9               |
| Alanine    | 8.3               |
| Methionine | 2.8               |
| Isoleucine | 3.0               |
| Leucine    | 8.0               |
| Tyrosine   | 0.9               |
| Phenylalanine | 2.1         |
| Tryptophan | 3.0               |

*This value for cysteine is based only on results obtained from analysis of hydrolysate.
1 Not analyzed.

(13) and with the results of analysis by Keutmann et al.4 of homogeneous hormone obtained by Cm-cellulose chromatography in which 8 M urea was incorporated into the eluting solution.

Adsorption of Parathyroid Hormone to Glassware and Apparatus—During the course of our preliminary studies it became apparent that when working with microgram quantities of parathyroid hormone such as we encountered in our experimental samples, a significant fraction of the parathyroid hormone could be lost from solution by adsorption to glassware and other apparatus. These losses made difficult the quantitation of synthesis of hormone in our tissue slice system. The nature and magnitude of this problem are illustrated by the following examples.

As much as 80% of a 10-μg sample of hormone could be adsorbed to the walls of ordinary borosilicate glass test tubes (Fig. 3). Adsorption required about 4 hours to reach maximum. There was no detectable adsorption of radioactivity when trichloroacetate powder, pure parathyroid hormone (not shown), or human serum was included in the sample, whereas crystalline bovine serum albumin and lysozyme, selected as representative proteins, were only partially effective. In separate tests, serum from a human subject with hyperparathyroidism also prevented adsorption, indicating that the parathyroid hormone component of normal serum was not the active agent in these tests. The adsorption of radioactive hormone could readily be reversed by adding trichloroacetate powder to an unadsorbed sample (Fig. 3, -/+ TCA). The hormone adsorbed to flint glass, polycarbonate, and cellulose nitrate to about the same degree as to borosilicate glass but to a lesser extent to polypropylene test tubes.

Adsorption of hormone to Sephadex chromatographic columns was next studied. When microgram quantities of 3H-parathyroid hormone (5 to 300 μg) were applied to unused Sephadex G-100 columns, the hormone was not eluted as a discrete peak. Instead, it was slowly leached from the column during passage of several column volumes of eluent. At this stage the adsorbed radioactive parathyroid hormone could not be released either by increasing the concentration of the eluting buffer to 1.0 M or by lowering the pH, or by both. At any time during this leaching process the application to the column of a large quantity of trichloroacetate powder (Wilson), led to the elution, as a discrete band, of the radioactive hormone previously unreleased. Fig. 4 illustrates this phenomenon. 3H-Parathyroid hormone (5 μg) containing 6800 dpm of radioactivity was applied to the column. During the passage of 1.7 column volumes of eluting buffer, there occurred only a slight increase in radioactivity over background which indicated that hormone was, in fact, undergoing elution. When at this point, 10 mg of trichloroacetate powder (Wilson), were applied to the top of the column and elution was continued, approximately 50% of the total radioactive sample emerged in the proper location as judged by the absorbancy pattern of the trichloroacetate powder (Wilson). It is interesting to note that the previously unreleased 3H parathyroid hormone emerged coincident with the protein component of the trichloroacetate powder which contains parathyroid hormone. This suggests that specificity is required of the protein carrier necessary for elution of the hormone.

The notion that “specificity” is required of the protein necessary for elution of parathyroid hormone is further shown in Fig. 5. Bovine serum albumin and lysozyme were tested in addition to trichloroacetate powder (Wilson). At the same protein concentrations, the first two proteins were much less effective.
In order to minimize adsorption of the radioactive hormone, the Sephadex columns could be "conditioned" prior to use by passing through them 5 mg of trichloroacetate powder (Wilson). When this was done, amounts of purified, radioactive parathyroid hormone in the range of 5 to 305 μg were recovered in increasing quantities as a discrete band as the amount of hormone applied was increased. Thus when the highest level of parathyroid hormone was used, essentially all of the radioactive hormone emerged as a single peak.

Studies similar to those outlined above with Sephadex, were also performed with Cm-cellulose columns and ³H- or ¹⁴C-parathyroid hormone was used, essentially all of the radioactive hormone emerged as a single peak.

Fig. 4. Absorption of ³H-parathyroid hormone (³H-PTH) in unused Sephadex G-100 chromatographic column and its subsequent elution. A 5-μg sample of ³H-parathyroid hormone containing 6800 dpm was applied to a freshly prepared Sephadex column. Elution was initiated with buffer system B and continued for 1.7 total column volumes (1.7 V₀). At B, 10 mg of trichloroacetate powder (Wilson) were applied and elution was continued. Fractions of 2.5 ml were collected. Aliquots of 2.0 ml each were sampled for assay of radioactivity. V₁ refers to the void volume of the column. The total radioactivity recovered in Fractions 1 to 173 was 1100 dpm, and in Fractions 174 to 184, 3300 dpm.

Fig. 5. Gel filtration on G-100 of radioactive parathyroid hormone in the absence and presence of other protein. A sample of 15 μg of ³H-parathyroid hormone (25,500 dpm) alone or mixed with 5 mg of the following proteins, +PTH, trichloroacetate powder (Wilson); BSA, bovine serum albumin; +LYSOZYME, was applied to "conditioned" Sephadex columns (see text). The columns were eluted with Buffer System B. Effluents were monitored at 280 μm where applicable and 2.0 ml aliquots were removed from each fraction for measurement of radioactivity.
concentration of calcium (Fig. 6B). Usually there was another major band of $^{14}$C-radioactivity which eluted shortly after the parathyroid hormone-containing band whose radioactivity was also greater in the medium containing the lower concentration of calcium. In contrast to the enhanced incorporation of $^{14}$C-leucine into this band and the parathyroid hormone-containing peak, there was little if any effect of low calcium on the incorporation of radioactivity into the larger protein components which eluted earlier from the columns. Fig. 7, A and B, illustrate the results of further chromatography on Cm-cellulose of the parathyroid hormone-containing bands obtained from Sephadex. In both cases there was a single major peak of $^{14}$C-radioactivity which exactly coincided with the added $^3$H-parathyroid hormone marker. No evidence was found for the presence of a band of radioactivity corresponding to that which emerged in Fractions 92 to 100 when tissue extracts were fractionated (Fig. 1B). Upon polyacrylamide electrophoresis, the newly synthesized $^{3}H$-parathyroid hormone comigrated with the marker $H$-parathyroid hormone. About 50% of the $^{14}C$-radioactivity in the Sephadex peaks which were applied to the Cm-cellulose columns was associated with parathyroid hormone as indicated by $H$-marker. The radioactive heterogeneity of these fractions was similar to that observed with the Sephadex-parathyroid hormone fractions obtained from tissue-trichloroacetate powders (see Fig. 1, A and B). The amount of incorporated radioactivity was 4-fold greater in the case of the low calcium sample than the high calcium sample (2,200 versus 550 dpm). The $H$-parathyroid hormone recovered from the Cm-cellulose column, for the aliquots applied were 12,000 and 13,400 dpm for the low and high calcium samples, respectively. Since 57,600 dpm of $H$-parathyroid hormone were added to each of the original samples of medium, the calculated total radioactivity of the newly synthesized parathyroid hormone found in the incubation medium under conditions of low and high calcium concentration were 10,560 and 2,260 cpm, respectively. The magnitude of this effect of calcium concentration was similar to that found for the incorporation of amino acid into parathyroid hormone isolated from the incubated tissue, itself, and indicates that changes in media hormone reflects similar changes in the tissue itself.

After correction for sampling, the total recovery of added marker for both samples was about 50%. This degree of loss was due to adsorption to glassware and other apparatus as described above and would have been much larger if trichloroacetate powder had not been added to the original samples of medium. In fact, in a series of experiments in which media samples were chromatographed without carrier, the losses were so severe that we were unable to obtain enough parathyroid hormone protein from the medium to measure chemically. A corollary to the requirement of adding carrier hormone is that radioimmunoassay of such samples has little meaning.

In order to be certain that the effect of calcium concentration on the amount of radioactive parathyroid hormone found in the medium was not due simply to changes in the rates of degradation of the radioactive hormone released in the medium, we studied the effect of calcium concentration on such degradation. Radioactive parathyroid hormone was incubated with slices for various periods of time under conditions identical to those used for biosynthesis. Fig. 8 shows that over a 6-hour period of incubation, calcium concentration had no effect on the amount of radioactive substrate parathyroid hormone recovered from the medium.

The time course of the incorporation of $^3$H-leucine into the protein of the media after incubation is shown in Fig. 9. The radioactivity in the peak containing parathyroid hormone (identified by the location of the internal $^{14}C$-parathyroid hormone marker) increased over the course of the 4-hour period of incubation. The width of these peaks, in contrast to the sharper marker peak, indicates the heterogeneous nature of the former. The band eluting after parathyroid hormone also increased beginning after the 1st hour of incubation and by the end of the 2nd hour contained the major fraction of the incorporated radioactivity. The incorporation of $^3$H-leucine into

![Fig. 6. Sephadex chromatography of incubation media from low and high calcium incubations. Incubation of parathyroid gland slices (1.75 g) was performed in 10 ml of buffer at a calcium concentration of 0.5 mM for A and 2.0 mM for B with 0.5 μCi per ml of $^{14}C$-leucine for 1 hour. The incubation medium was quickly chilled and centrifuged. Trichloroacetate powder (Wilson, 5 mg), protein, and 17 μg of $^3H$-parathyroid hormone (57,600 dpm) were added to the supernatant fluid. Of this mixture 7.5 ml were applied to a Sephadex G-100 column and elution was carried out with Buffer System B. For this experiment the volume per fraction was 3.2 ml for A and 2.5 ml for B. Absorbance of eluent was measured and aliquots from each tube were taken for measurement of radioactivity. For ease of presentation of data only the leading edge of the large band of free radioactive leucine is portrayed in the figure. The parathyroid hormone-containing fractions were pooled as follows. A, Fractions 32 to 37 ($^{14}C$ 9.25 X 10$^3$ dpm; $^{14}C$ 8.1 X 10$^3$ dpm). B, Fractions 41 to 47 ($^{14}C$ 3.4 X 10$^3$ dpm; $^{14}C$ 3.1 X 10$^3$ dpm). These fractions were lyophilized and dissolved in 2 ml of 0.01 M ammonium acetate buffer, pH 5.3, for chromatography on Cm-cellulose as shown in Fig. 7.

![Diagram](image-url)
Studies on Biosynthesis in Vitro of Parathyroid Hormone. II

Fig. 7. Cm-cellulose chromatography of Sephadex-purified media parathyroid hormone fractions. Aliquots (1.5 ml) of the solution of the Sephadex-PTH fractions noted in Fig. 6 were chromatographed as described under "Methods." High Ca++, sample B from Fig. 6B. The parathyroid fraction (44 to 51) obtained from the Cm-cellulose column contained 13,400 dpm of 3H and 550 dpm of 14C. Low Ca++, sample A from Fig. 6A. The parathyroid fraction (39 to 45) obtained from the Cm-cellulose column contained 12,000 dpm of 3H and 2200 dpm of 14C.

Fig. 8. Degradation of 14C-parathyroid hormone. Flasks containing 1.75 g of slices were previously incubated in 10 ml of buffer at a calcium concentration of 1.25 mM for 1 hour. Tissues were then rinsed and transferred to fresh media containing 15 mM of 14C-parathyroid hormone (5,000 dpm) and either 0.5 mM calcium (•) or 2.0 mM calcium (○). Incubation was then continued for the periods indicated. Media from the incubations was then mixed with 10 μg of 3H-parathyroid hormone (2000 dpm per μg) as marker and 5 mg of trichloroacetate powder (Wilson) as carrier and chromatographed on Sephadex G-100 with Buffer System B. The tubes corresponding to marker were pooled and assayed for radioactivity. The 14C-radioactivity was corrected for recovery of marker 3H-parathyroid hormone. Data are presented as total 14C-disintegrations per min remaining as ungraded parathyroid hormone in the sample.

Cm-cellulose-purified parathyroid hormone of the medium is shown in Fig. 10. The total amount of radioactivity in the hormone increased linearly with time. Also shown in the figure is the relative immunoreactivity of the media to parathyroid hormone antiserum. The 30-min sample was arbitrarily assigned a value of 1 and the values for the other samples are reported relative to the 30-min sample. On the basis of relative immunological activity there was an apparent rapid release of a large amount of pre-existent (nonradioactive) immunoreactive material followed by a slower and possibly declining rate of release.

Fig. 9. The time course of incorporation of 3H-leucine into media protein. Portions (4 g) of gland slices were incubated in 30 ml of buffer containing 5 μCi per ml of 3H-leucine (0.5 mM Ca++) for the indicated number of hours (arrows). Aliquots were removed for radioimmunoassay at the end of incubation after which 23,100 dpm of 14C-parathyroid hormone (14C-PTH) were added as internal standard. Trichloroacetate powder (Wilson), was not added as carrier. The media were then chromatographed on Sephadex G-100 (Buffer System B). For ease of presentation of data, the pattern of radioactivity of the internal standard is shown for the 1-hour sample only; the general shape and the elution volume for the peak of radioactivity of each of the internal standards were identical for all four columns. Aliquots (1.0 ml) were removed from the fractions for measurement of radioactivity. In each case Fractions 41 to 50 were pooled and lyophilized for Cm-cellulose chromatography.

Fig. 10. Time course of appearance of immunological activity and incorporation of 3H-leucine into newly synthesized parathyroid hormone (PTH). The parathyroid hormone-containing fractions from Fig. 9 were chromatographed on Cm-cellulose in order to isolate parathyroid hormone. The data on incorporation of 3H-leucine into parathyroid hormone represents the total in each incubation medium and was obtained by correcting for recovery of internal standard. The radioimmunoassay was performed on aliquots of the original incubation media before adding the internal standard (14C-parathyroid hormone) and is presented as Relative Immunoreactivity with 30-min sample taken as a potency of 1.
discharge. In view of the uncertainty of the absolute amount of parathyroid hormone in the media and the inability to obtain enough isolated product for protein analysis, a determination of the specific activity of the newly synthesized hormone was precluded.

In order to determine whether or not other radioactive fractions in the Sephadex elution profiles of the medium were immunoreactive with parathyroid hormone-antiserum, several pooled fractions throughout the profiles shown in Fig. 9 were tested. Each of the profiles was cut as follows: Fractions 25 to 40, 41 to 50 (PTH), and 51 to 60. About 97% of the immunoreactive material eluted from the column resided in the parathyroid hormone-containing fraction; the remaining 3% was distributed equally in the leading and trailing edges of the parathyroid hormone peak. This pattern was unaffected by the period of incubation of the tissue, from 0.5 to 4 hours.

**Reversible Nature of Effect of Calcium on Biosynthesis**—That the inhibitory effect of high concentrations of calcium on parathyroid hormone biosynthesis in the tissue slice system was reversible is shown by the experiment illustrated in Fig. 11. Tissue slices were first incubated for 1 hour in buffer containing 2.0 mM (Tissue A) or 0.5 mM (Tissue B) calcium together with 3H-leucine (Phase I). After rinsing, half of the tissue was removed and analyzed for incorporation into parathyroid hormone (isolated through the Sephadex stage of purification). The other half of the tissue was rinsed and placed in fresh buffer containing 3H-leucine and a calcium concentration of 0.5 mM (Tissue A) and 2.0 mM (Tissue B) and incubated for an additional hour (Phase II), after which the tissue was removed and analyzed for incorporation into parathyroid hormone (isolated through the Sephadex stage of purification). The other half of the tissue was removed and analyzed for incorporation into parathyroid hormone (isolated through the Sephadex stage of purification). The data show that the amount of radioactive amino acid incorporated into parathyroid hormone was inversely related to the calcium concentration during both the first and second phases of the experiment.

**Lack of Effect of Magnesium on Biosynthesis of Parathyroid Hormone**—The recent report which implicates magnesium in regulation of parathyroid hormone secretion by the gland (5, 15) both in vivo and in vitro led us to test the effect of this substance on de novo biosynthesis by the gland slices. Table II shows the effect of a wide difference in magnesium concentration in the presence of a constant calcium concentration to the usual conditions of a low and high calcium concentration in the presence of a fixed magnesium concentration. Under our experimental conditions, extremes of magnesium concentration (0.2 to 1.7 mM) were without effect on parathyroid hormone biosynthesis. In contrast, there was a doubling of specific radioactivity of parathyroid hormone and the total amount of radioactivity as parathyroid hormone found in the gland at the end of the incubation under conditions of low calcium incubation as compared with high. There was no difference in total amount of hormone in tissue in these studies. Likewise, although not shown in the table, there were no statistical differences in immunoreactivity of the medium to parathyroid hormone antiserum.

![CALCIUM (mM)](image)

**Fig. 11.** Reversible effect of calcium on the biosynthesis of parathyroid hormone in parathyroid gland slices. A and B refer to the corresponding tissue sample in both phases of the experiment. The numbers in the bars denote the concentration of calcium in the buffer in which the tissue was incubated. Other details are in the text.

**TABLE II**

| Buffer composition | Parathyroid hormone in tissue |
|--------------------|-------------------------------|
| Calcium            | Magnesium | Specific activity (dpm/µg parathyroid hormone) | Total parathyroid hormone (dpm/g) | Total 3H-parathyroid hormone incorporation (dpm/g) |
|                    | mM        |                                   | µg/g gland |                               |                               |
| Experiment A       |           |                                    |             |                               |                               |
| 0.5                | 0.83      | 108                                 | 172         | 18,600                        |
| 2.0                | 0.83      | 50                                  | 192         | 9,600                         |
| 1.25               | 0.21      | 62                                  | 150         | 9,300                         |
| 1.25               | 1.67      | 69                                  | 169         | 11,700                        |
| Experiment B       |           |                                    |             |                               |                               |
| 0.5                | 0.83      | 400                                 | 39          | 15,600                        |
| 2.0                | 0.83      | 180                                 | 29          | 5,200                         |
| 1.25               | 0.21      | 325                                 | 31          | 10,100                        |
| 1.25               | 1.67      | 318                                 | 34          | 10,800                        |

**DISCUSSION**

The present study extends our previous report (4) which showed that bovine parathyroid gland slices incubated *in vitro* synthesize parathyroid hormone, that the degree of incorporation

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1 Preliminary studies using radioactive parathyroid hormone in isotope dilution studies with fresh bovine parathyroid gland tissue and acetone powders indicate that the content of parathyroid hormone in these preparations is somewhat greater than previously thought.

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**TABLE I**

| Buffer composition | Parathyroid hormone in tissue |
|--------------------|-------------------------------|
| Calcium            | Magnesium | Specific activity (dpm/µg parathyroid hormone) | Total parathyroid hormone (dpm/g) | Total 3H-parathyroid hormone incorporation (dpm/g) |
|                    | mM        |                                   | µg/g gland |                               |                               |
| Experiment A       |           |                                    |             |                               |                               |
| 0.5                | 0.83      | 108                                 | 172         | 18,600                        |
| 2.0                | 0.83      | 50                                  | 192         | 9,600                         |
| 1.25               | 0.21      | 62                                  | 150         | 9,300                         |
| 1.25               | 1.67      | 69                                  | 169         | 11,700                        |
| Experiment B       |           |                                    |             |                               |                               |
| 0.5                | 0.83      | 400                                 | 39          | 15,600                        |
| 2.0                | 0.83      | 180                                 | 29          | 5,200                         |
| 1.25               | 0.21      | 325                                 | 31          | 10,100                        |
| 1.25               | 1.67      | 318                                 | 34          | 10,800                        |

**DISCUSSION**

The present study extends our previous report (4) which showed that bovine parathyroid gland slices incubated *in vitro* synthesize parathyroid hormone, that the degree of incorporation
of radioactive amino acid into parathyroid hormone is inversely related to the calcium concentration of the incubation medium, and that this effect of calcium is directed towards biosynthesis of parathyroid hormone to a greater extent than to general proteins of the tissue.

Under conditions of low calcium concentration there was an increased specific activity of parathyroid hormone and a major increase in total amount of amino acid incorporated into the hormone, but little difference in amount of total hormone in the gland slices at the end of the incubation period (Table II). It is important to note that a change in specific radioactivity of the hormone would not alone be sufficient to conclude that a corresponding change in net synthesis had occurred. For example, an increase in the specific radioactivity of parathyroid hormone would also result from a situation in which net synthesis of hormone did not change, but loss of stored hormone from the tissue increased. Alternatively, an increase in the specific radioactivity of a pool of precursor to parathyroid hormone would yield similar data. A number of kinetic models in which net input and output of hormone were varied together or independently were considered. The explanation which best fits our finding of both an increase in specific activity and total incorporation of radioactivity into parathyroid hormone with little change in total tissue hormone is that net synthesis increased when the calcium concentration was lowered. The fact that the slices retained their responsiveness to calcium after having been cycled through both high and low concentrations of calcium (Fig. 11) tends to rule out the possibility that there was an increase in specific activity of a precursor pool, due, for example, to a change in permeability of the slices with resultant loss of cellular constituents to the medium. The present findings, together with our earlier determination that the specific activity of the tissue pool of free radioactive amino acid was unaffected by calcium concentration, support the conclusion that the net biosynthesis of parathyroid hormone in the tissue slice system was, in fact, enhanced at low concentration of calcium.

A portion of this newly synthesized hormone was released into the incubation medium in an amount which also varied inversely with calcium concentration. This result could not be accounted for solely on the basis of a differential rate of degradation of released hormone since the concentration of calcium in the incubation medium did not appear to significantly affect the rate of degradation of parathyroid hormone added to the system (Fig. 8).

Since the basis for selection of a fraction of medium for further purification of the parathyroid hormone was based on its correspondence with authentic marker parathyroid hormone, we may not eliminate the possibility that other species of hormone or immunologically reacting molecules were present in the incubation media. Indeed, this may prove to be the case in light of our observation that the immunological activity of the whole incubation medium differed from that of the tissue hormone. Furthermore, although the bulk of immunological reactivity of medium Sephadex profiles was found at an elution volume which corresponded to the region where authentic parathyroid hormone eluted, there was immunological activity found preceding and following this major peak. The possible existence of other hormonal species secreted by the parathyroid gland is reinforced by the earlier finding of Berson and Yalow (16) of an immunological heterogeneity of circulating parathyroid hormone; the study of Aunaud, Tsao, and Oldham (17) which demonstrated a difference in immunological reactivity between parathyroid hormone extracted from human parathyroid adenoma and that secreted in an organ culture of the tissue; and the recent report of Sherwood et al. (14) that bovine parathyroid glands maintained in culture secreted a species immunologically different and somewhat smaller in size than that obtained from tissue extracts. On the other hand, immunological reactivity is not alone sufficient to conclude that a species of biologically functional hormone is in fact present. Thus, Potts, Aurbach, and Sherwood (8) showed that chemical alterations of the native molecule could result in a species with full immunological activity and little or no biological activity. Therefore, it is important that alternate means of identification including isolation of species, such as performed herein, be accomplished.

Our observation that changes in magnesium concentration had no effect on biosynthesis of parathyroid hormone is of importance in light of the increasing evidence that secretion of parathyroid hormone is stimulated at low concentrations of either magnesium or calcium (5, 15). One possible explanation of these findings is that whereas calcium affects, directly or indirectly, both synthesis and secretion of the hormone, magnesium affects only secretion. We were unable to test this hypothesis in our present system since we could not find statistically significant differences in the amount of immunoreactive parathyroid hormone in the medium over the range of either calcium or magnesium concentrations tested. At present, we are assessing whether or not the tissue slice system can serve as a definitive model for studies on secretion of hormone as well as its use as a system for study of biosynthesis. There is reason to believe that the system with suitable modification might serve the former function.

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