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STRUCTURAL BASIS OF BIOLOGICAL AND IMMUNOLOGICAL ACTIVITY OF PARATHYROID HORMONE

BY JOHN T. POTTS, JR.,* G. D. AURBACH,† LOUIS M. SHERWOOD,* AND A. SANDOVAL*

NATIONAL HEART INSTITUTE AND NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES

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We have obtained sufficient information to propose a model of the structure of parathyroid hormone including the partial sequence of a COOH-terminal region, biologically and immunologically active, that constitutes only 25 per cent of the native molecule. This communication shows how this structure was deduced through survey experiments analyzing a series of chemical derivatives and peptide fragments prepared to study the amino acid sequence of the polypeptide.[1, 2]

Materials and Methods.—Extraction and purification: The hormone was extracted from bovine parathyroid glands with phenol,[3, 4] fractionated with salt and trichloroacetic acid (TCA-PTH),[4] and purified by gel filtration on Sephadex G-100 (Sephadex-PTH)[5] and chromatography on

carboxymethylcellulose (CMC-PTH).[1, 2] Biological activity was assayed by a modification[6] of the method[7] of Munson; the potency of the final product was 2000–3000 USP units/mg of protein.

Amino acid analysis: Amino acids were analyzed on Beckman-Spinco model 120B instruments, modified for high-sensitivity detection by use of dual range recorders (0–5 and 4–5 millivolt) and 6.6-mm light path cuvettes.[8] Under these conditions, samples of 0.0025–0.2 μmole could be analyzed with a reproducibility of 4%. Acid hydrolysis was carried out with 6 *N* HCl in sealed, evacuated tubes for 20–24 hr; for alkaline hydrolysis 5 *N* NaOH was used.[9] Tryptophan was analyzed using a column packed to a height of 7 cm with Beckman-Spinco PA-35 resin, and developed with the pH 5.2 sodium citrate buffer containing n-propanol.[10]

Cleavage of the polypeptide by chemical and enzymic methods: Carboxypeptidase, leucine aminopeptidase, trypsin, and pepsin were obtained from Worthington Biochemical Co. The exopeptidases, treated with an excess of diisopropylfluorophosphate to destroy endopeptidase activity, were tested for specificity as described previously.[11] Incubations for periods up to 72 hr were carried out with carboxypeptidase and leucine aminopeptidase using the volatile buffers, ammonium bicarbonate and trimethylamine acetate, respectively. The rate of release of amino acids was determined with aliquots of the digest mixtures applied directly to the analyzer; correction was made for amino acids liberated by autodigestion (determined by analysis of control solutions containing the enzyme alone). Digestion with trypsin was monitored with a radiometer pH stat using 0.05 *M* trimethylamine as titrant; 0.1 *M* ammonium formate, pH 2.8, was the buffer for incubations with pepsin. Hormone concentrations of 2–10 mg/ml were used with 0.1–0.2 mg/ml of the enzymes. Reaction with cyanogen bromide was used as described by Gross and Witkop;[12] the method of dilute acid hydrolysis (0.03 *N* HCl at 110° for 12 hr) followed the procedure of Tsung and Fraenkel-Conrat.[13] Polypeptide fragments of the hormone produced by these procedures were isolated by peptide mapping;[14] for separations on a preparative scale 0.3–0.4 μmoles of peptide digest were applied to the Whatman #3 paper.

Preparation and testing of derivatives of the hormone: The hormone was treated with N-bromosuccinimide or 2-hydroxy-5-nitrobenzyl bromide by the techniques described for these reagents;[15, 16] modification of tryptophan was determined by amino acid analysis after alkaline hydrolysis. Tyrosinase (the gift of Dr. C. R. Dawson) was incubated with the hormone (2 mg/ml) in 0.05 *M* ammonium bicarbonate; the loss of tyrosine due to oxidation was determined by analysis of the derivative hydrolyzed in acid. Hydrolysis of the enzyme used did not yield amounts of amino acids sufficient to interfere with analysis of the hormone derivative. Performic acid was prepared by reacting 19 vol of 98% formic acid with 1 vol of 30% hydrogen peroxide for 1 hr at room temperature; 1 ml of the mixture was added to 1–3 mg of hormone, kept at 0°C for 2 hr, then diluted with distilled water at 0° and lyophilized. Analysis of these hormone derivatives after acid hydrolysis showed that amino acids other than those intentionally modified were unaltered. In certain preliminary experiments, the hormone was esterified by reaction with methanol-HCl or acetylated with acetic anhydride.

Bioassays were performed on samples of the derivatives before and after reduction with cysteine to ensure that any loss of activity had not been brought about by oxidation of methionines during the reaction.[17, 18] Immunological activity was measured by the method of Berson *et al.*;[19] this radioimmunoassay is based on the competitive inhibition by unlabeled hormone of the binding of I^{131}-labeled hormone to specific antibody. Mixtures of antibody and I^{131}-labeled hormone were incubated with unknown or standard hormone preparations for 2–6 days; the mixtures were then fractionated by chromatoelectrophoresis, a technique separating antibody-bound hormone (*B*) from free hormone (*F*). The concentration of hormone in an unknown sample was obtained from graphs prepared by plotting the ratio of bound hormone to free hormone (*B*/*F* ratio) as a function of the concentration of standard hormone. Derivatives of the hormone with reduced immunological reactivity (less effective in displacing I^{131}-labeled hormone from antibody) showed, on graphical analysis, curves (*B*/*F* vs. concentration) that differed in slope from that produced by the native hormone. For measurement of hormone in plasma, antiserum was diluted to 1/100,000 or greater;[20] similar dilutions were used to test the derivatives. The interaction between certain antigenic sites in parathyroid hormone and the antibodies directed against these sites might not be apparent at this marked dilution.[20] However, the primary purpose here was to determine the region of the molecule necessary to react with antibody under the conditions used for radioimmunoassay of the hormone in plasma.[21, 22]

Results.–Parathyroid hormone purified sequentially on Sephadex G-100 and CM-cellulose was devoid of detectable nonhormonal polypeptide. However, two forms of the hormone could be distinguished by disc gel electrophoresis of CMC-PTH at pH 2.8 in 8 *M* urea. Analysis indicated that the two forms separable, so far, only by the disc gel technique[23] were biologically, immunologically, and chemically similar.[24] These studies indicated that CMC-PTH was sufficiently pure to undertake studies of the structural basis of biological and immunological activity.

The covalent sequence of the hormone: Amino acid analysis was performed on samples of the hormone hydrolyzed for varying periods of time. The composition obtained was lysine_{10}, histidine_4, arginine_5, aspartic acid_8, serine_6, $\text{glutamic acid}_{10}$, proline_3, glycine_4, alanine_7, valine_7, methionine_2, isoleucine_3, leucine_8, tyrosine_1, phenylalanine_2, and tryptophan_1. Serial analyses made during repeated incubations of the polypeptide with either carboxypeptidase or leucine aminopeptidase indicated the probable order of amino acids at each terminal portion of the molecule. These studies showed that the single tryptophan and tyrosine and the two methionines and phenylalanines, each pivotal markers for the sequence study, were located peripherally. The presumptive sequence from the exopeptidase data at the amino-terminus was:

H·Ala·Val·Ser (Glu, Gly, Leu, Lys, His, Ileu, Met, Phe)

and at the COOH-terminus:

(Tyr, Gly, Arg) Tryp·Lys·His·Ileu·Met·Glu·Ser·Phe·Ala·Val·Leu·Gn·OH

Analysis with the pH stat showed that digestion of parathyroid hormone by trypsin was essentially complete within 45 min; however, less than 50 per cent of the potentially susceptible bonds (at the carbonyls of the 10 lysines and 5 arginines) in the molecule were cleaved. Only 7 major peptides were detected as shown in Figure 1; many of the fragments found by peptide mapping after 1 hr of digestion persisted throughout 24 hr of incubation, confirming that these peptides were resistant to attack by trypsin. The composition of each peptide isolated from the peptide maps is shown in Table 1. These results indicated that basic amino acids were clustered in certain areas (explaining the resistance of these regions to further cleavage by trypsin). The sum of amino acids found in tryptic peptides T1 to T7 accounted for the amino acid content of the hormone except for 10 residues (Table 1). After cleavage of the hormone by cyanogen bromide, a large peptide fragment 55 amino acids in length (representing the portion of the molecule interior to the methionines) was obtained by precipitation with trichloroacetic acid. This fragment contained the single tryptophan and tyrosine as predicted from the exopeptidase studies. Four smaller fragments representing the regions of the molecule peripheral to the methionines were isolated from the fraction soluble in trichloroacetic acid. Information derived from this study is illustrated in Figure 2; peptide C_3 represents the COOH-terminus and C_2 the amino-terminus of the molecule. Peptides C_1 and C_4 were presumably the products of secondary weak acid cleavage of C_2.

Products of the hormonal polypeptide hydrolyzed with dilute acid or digested with pepsin were also isolated by peptide mapping and analyzed for amino acid composition (Fig. 2).

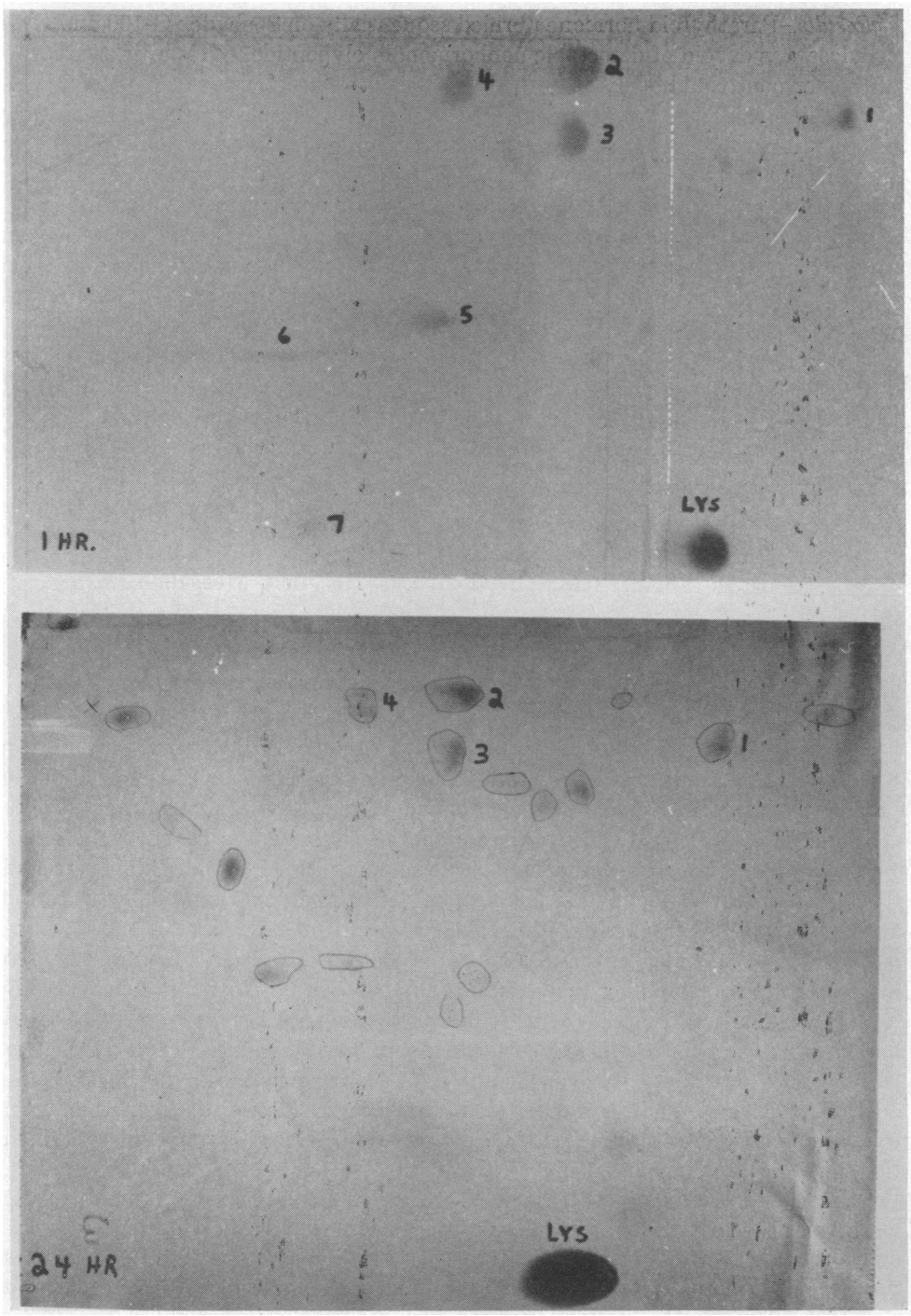

FIG. 1.—Two-dimensional separation of peptides produced after 1 or 24 hr of digestion of parathyroid hormone with trypsin. The principal peptides 1, 2, 3, and 4 found at 1 hr persisted with little further degradation throughout the 24 hr of incubation. Lysine (*LYS*) served as reference for electrophoretic migration.

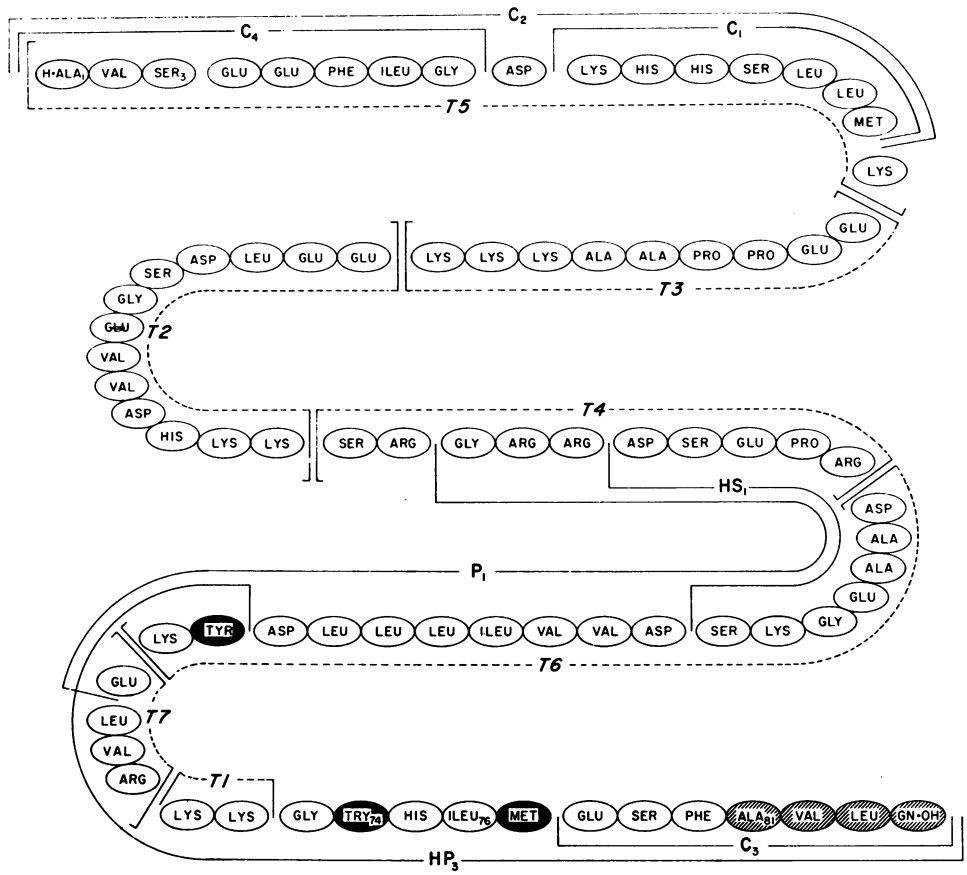

FIG. 2.—Proposed model for the structure of parathyroid hormone deduced from studies with exopeptidases and the composition of fragments produced by cyanogen bromide cleavage (*C*), dilute acid hydrolysis (*HP* and *HS*), and digestion with pepsin (*P*). The regions corresponding to each of the tryptic peptides (*T1* through *T7*) are outlined by the parentheses and dotted lines; absolute sequence within these regions is not known but the individual residues are arranged to reflect the partial sequence information deduced from exopeptidase studies, the characteristic specificity of tryptic attack, and the composition of fragments produced by other means of cleavage. Peptide C_2 represents the amino-terminal portion and peptide C_3 the carboxyl-terminal portion of the molecule (peptides C_1 and C_4 arose through secondary weak acid hydrolysis of C_2). The composition of C_3 fits the analysis for this region obtained by digesting the hormone with carboxypeptidase. The analysis of P_1 corresponds exactly to the sum of tryptic peptides *T4*, *T6*, and part of *T7*.

The cross-hatched residues at the COOH-terminus were not necessary for activity. The 20-amino acid region (HP_3, the fragment cleaved from parathyroid hormone by dilute acid hydrolysis) at the carboxyl terminus was biologically and immunologically active. Alteration of methionine, tryptophan, or tyrosine (shown by symbols with darkened borders), also located within HP_3, caused marked loss of biological activity.

Relationship of the structure of parathyroid hormone to its biological and immunological activity: A number of modifications of the hormone were made; results of tests of the activity of these derivatives are listed in Table 2.

Removal of the four carboxyl-terminal amino acids—glutamine, leucine, alanine, and valine—from parathyroid hormone by digestion with carboxypeptidase did not inactivate the molecule. However, further digestion to remove phenylalanine and/or the next four to five amino acids toward the interior caused marked inactivation, as illustrated in Table 2. Hydrolysis with dilute acid caused extensive cleavage of

TABLE 1

AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF PARATHYROID HORMONE

	Peptides							Sum T1 to T7	Total in PTH
	T1	T2	T3	T4	T5	T6	T7		
Lysine	2	2	3	0	2	2	0	11	10
Histidine	..	1	0	0	2	0	0	3	4
Arginine	..	0	0	4	0	0	1	5	5
Tryptophan	..	..	..	..	..	..	..	0	1
Aspartic acid	..	2	0	1	1	3	0	7	8
Threonine	..	0	0	0	0	0	0	0	0
Serine	..	1	0	2	2	1	0	6	6
Glutamic acid	..	3	2	1	2	1	1	10	10
Proline	..	0	2	1	0	0	0	3	3
Glycine	..	1	0	1	1	1	0	4	4
Alanine	..	0	2	0	1	2	0	5	7
Cystine (half)	..	0	0	0	0	0	0	0	0
Valine	..	2	0	0	1	2	1	6	7
Methionine	..	0	0	0	1	0	0	1	2
Isoleucine	..	0	0	0	1	1	0	2	3
Leucine	..	1	0	0	2	3	1	7	8
Tyrosine	..	0	0	0	0	1	0	1	1
Phenylalanine	..	0	0	0	1	0	0	1	2
Residues/peptide	2	13	9	10	17	17	4	72	80

A tryptic peptide representing the carboxyl-terminus (containing one histidine, alanine, valine, methionine, phenylalanine, isoleucine, and leucine) was not detected on the peptide maps. However, the composition of this area could be deduced from the studies of fragments made by reaction with cyanogen bromide, hydrolysis with dilute acid, and digestion with carboxypeptidase.

TABLE 2

EFFECTS OF SELECTIVE MODIFICATION OF PARATHYROID HORMONE

Enzyme or chemical reagent	Modification	Biological activity (%)	Immunological activity
2-OH,5 nitrobenzyl bromide	1/1 Tryptophan reacted	30	Complete
n-Bromosuccinimide	1/1Tryptophan oxidized	10	Complete
Performic acid	2/2 Methionines 1/1 Tryptophan oxidized	0	Complete
Tyrosinase	0.8/1 Tyrosine oxidized	15	Weakly reactive
Methanol HCl	Carboxyl groups esterified	20	Weakly reactive
Acetic anhydride	Amino groups acetylated	0	Inactive
Carboxypeptidase	100% Removal of 4 C-terminal amino acids;	80	Complete
	80% removal of 4 residues beyond the 4 C-terminal amino acids	20	Strongly reactive
Dilute HCl	Cleavage of 70% of aspartyl peptide bonds	33	Strongly reactive
Cyanogen bromide	Cleavage removing 22 amino acids peripheral to the methionines	0	Strongly reactive
Trypsin	Cleavage into eight polypeptide fragments	0	Weakly reactive

aspartyl peptide bonds (70% of the aspartic acid of the polypeptide was detected as the free amino acid). Despite this extensive modification (native hormonal polypeptide was no longer detectable by disc gel electrophoresis or two-dimensional peptide mapping) 30 per cent of the biological and most of the immunological activity was retained in the weak acid hydrolysate. The activity was associated with only one of the fragments (peptide HP_3 in Fig. 2) identified on the peptide maps of the acid-cleaved hormone. The method for immunological testing is illustrated by Figure 3: this compares the reactivity of unmodified parathyroid hormone and several of its derivatives.

Discussion.–By combining the information gained from the exopeptidase studies with the composition of the peptide fragments resulting from cleavage of the

hormone with dilute acid, cyanogen bromide, and pepsin, it was possible to arrange the tryptic peptides in order (Fig. 2) beginning with peptide T5 assigned position #1 at the amino-terminus (however, the true order of tryptic peptides T2 and T3 might be the reverse of that shown). The outline shown in Figure 2 was designed only as a working model of the structure, but the close agreement between the results obtained by several methods of analysis suggested that the proposed structure was essentially correct. Although the internal sequence of most of the tryptic peptides is unknown, partial internal sequence could be deduced from exopeptidase studies, the composition of fragments derived by other means of cleavage, and the grouping of basic residues that must explain the resistance of certain fragments to further digestion by trypsin.

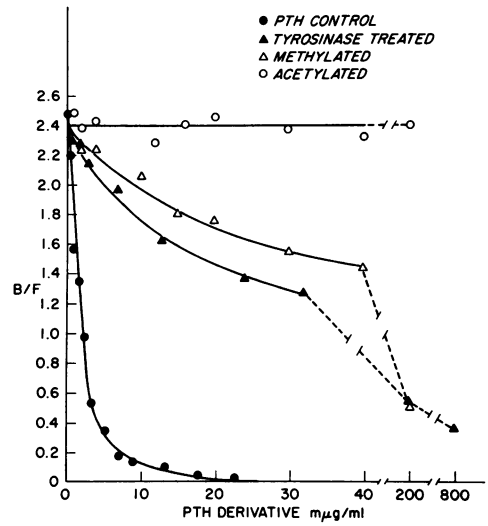

FIG. 3.—Graphical analysis of the immunological reactivity of modified parathyroid hormone preparations. *B/F* = ratio of hormone bound by antibody/free hormone. *PTH control* = reactivity of unaltered hormone. The tyrosinase-treated and methylated preparations were only weakly reactive; the acetylated derivative was inactive.

From the data in Table 2, it may be concluded that oxidation of tyrosine and oxidation or modification of tryptophan caused marked inactivation; the loss of activity brought about by oxidation of methionine has been previously described.[17, 18] Acetylation of the hormone destroyed both biological and immunological activity; methylation of the carboxyl groups of the hormone or oxidation of the tyrosine markedly reduced these activities. Marked divergence of immunological and biological activity occurred after modification of either tryptophan or methionine; immunological activity of these derivatives was little changed, whereas biological activity was greatly reduced. Another dissociation between biological and immunological activity was found after more extensive digestion of the molecule by carboxypeptidase (Table 2).

Hydrolysis of parathyroid hormone in weak acid proved the most useful means to define the minimum structure requisite for biological and immunological activity. This fragment, constituting 20 amino acids at the COOH-terminus (HP_3 of Fig. 2) contained one methionine, tyrosine, phenylalanine, and tryptophan but no proline. Since there was no aspartic acid in this region of the molecule, much of it remained intact during weak acid hydrolysis; the remainder of the molecule, not absolutely required for activity and rich in aspartic acid, was extensively degraded. The four COOH-terminal amino acids indicated by the cross-hatching seemed unimportant for biological activity; hormonal derivatives (treated with carboxypeptidase) lacking these residues were active. On the other hand, modification of particular amino acids within the HP_3 segment, the methionine, tryptophan, and tyrosine (shown in Fig. 2 with darkened borders), or extensive digestion with carboxypeptidase (removing the fifth through ninth residues at the COOH-terminus)

caused marked inactivation, establishing further the biological importance of this area of the molecule. The results with carboxypeptidase digestion suggested that if, in future experiments, the four carboxyl-terminal residues could be removed from the active fragment, one might obtain a peptide only 16 amino acids in length that would be biologically active. Since the hormonal derivatives resulting from treatment with cyanogen bromide or carboxypeptidase were still immunologically active, it was possible that a peptide even smaller than the biologically active fragment might be immunologically active. It is unknown whether even smaller active fragments could be obtained; however, it is known that the fragmentation of the carboxyl-terminal region of the molecule produced by digestion of the native hormone with trypsin destroyed biological activity completely.

Summary.—The amino acid composition of parathyroid hormone and its component tryptic peptides showed that the molecule was quite basic; lysine and arginine were clustered in certain areas, giving these regions a highly positive charge. The analysis of the covalent sequence is incomplete but the current information provided a useful model of the structure. A minimum structure requisite for biological activity appeared to reside in approximately 25 per cent of the molecule, a sequence 20 amino acids long at the COOH-terminus of the polypeptide chain. A slightly smaller segment in the same region was important for immunological reactivity. Prolonged digestion of the native hormone by carboxypeptidase or selective chemical alteration of the methionine, tryptophan, or tyrosine located within the carboxyl-terminal region caused marked loss of biological activity, but immunological activity was seriously impaired only by oxidizing the tyrosine. It is likely that the complete covalent sequence of parathyroid hormone will be available shortly; this information may allow one to correlate further the chemical structure of the hormone with its biological actions.

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* Section on Molecular Diseases, Laboratory of Metabolism, National Heart Institute.

† Section on Mineral Metabolism, Metabolic Diseases Branch, National Institute of Arthritis and Metabolic Diseases.

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*A POLYCISTRONIC MESSENGER RNA ASSOCIATED WITH β-GALACTOSIDASE INDUCTION**

BY YUKIO KIHO AND ALEXANDER RICH

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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A polycistronic messenger RNA molecule is one which contains information for more than one polypeptide chain. This implies that during the process of protein synthesis, more than one protein can be assembled on the same polyribosomal structure. It is frequently assumed that the induction of enzymes in bacterial systems is carried out through the formation of a polycistronic messenger RNA since the addition of an inducer substance is associated with the coordinated production of several protein molecules.[1] Several laboratories have demonstrated the formation of large-molecular-weight RNA molecules associated with bacterial enzyme induction and this is consistent with the interpretation of a polycistronic messenger molecule.[1-5] However, these experiments do not prove that a polycistronic messenger molecule is actually used during protein synthesis. For example, in mammalian cells, some large RNA molecules are broken down into smaller units before they are actually utilized.[6] Thus a more direct test is needed to be certain that a messenger RNA is polycistronic. We have chosen to demonstrate this using the polyribosomes on which the actual synthesis of proteins is occurring.[7] β-Galactosidase induction in *E. coli* is associated with the formation of three enzymes, β-galactosidase, galactoside permease, and thiogalactoside transacetylase.[8] It is possible to detect the presence of nascent β-galactosidase molecules attached to ribosomes and polysomes.[9-11] The size of the polysome is related to the size of the messenger RNA. Organisms which have a genetic deletion in the permease-acetylase region have smaller β-galactosidase polysomes than those seen in the wild type. From this we infer that the messenger RNA is polycistronic in this case.

Materials and Methods.—The general method used for preparing polysomes from *E. coli* has been described previously.[11] It involves pelleting penicillin spheroplasts, gently resuspending, and