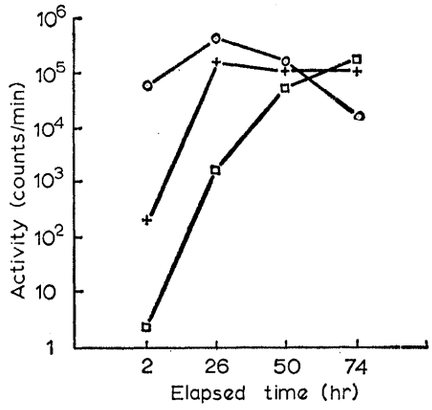

Fig. 1. Release of $C^{14}O_2$ by respiration at various periods after introduction of C^{14}-labeled glucose to mycelium. Carbon-14 activity is expressed as counts per minute per 0.5-ml sample of NaOH. Curves: +—+, average of four replicates of 11-week-old cultures of *P. tinctorius;* □—□, average of two replicates of 5½-week-old cultures of *P. tinctorius*; and ○—○, average of two replicates of 13-week-old cultures of *T. terrestris.*

agar and a small amount was probably lost upon neutralization.

The results of experiment 2 (Table 1) indicate highly significant amounts of activity in samples of all the cultures we tested. In cultures of *P. tinctorius*, samples of agar also showed the presence of C^{14}, although considerably less than in samples of mycelium plus agar. This may have been the result of diffusion of C^{14} in the agar after the C^{14} was absorbed by mycelium, but, more probably, it was a result of direct absorption of $C^{14}O_2$. The peak of activity in the 24- to 48-hour period and the decline with time was unexpected, since samples represent accumulative absorption of $C^{14}O_2$ up to the time of removal. This may have been caused by an initial concentration of C^{14} at the mycelium surface followed by an equilibrating diffusion throughout the substrate, but it would not explain the decrease in activity of the agar samples.

Representative cultures, other than those actually sampled in the experiments, were used to obtain an approximation of the weights of fungi in sample cores. In seven equivalent samples of *P. tinctorius*, the average dry weight was 1 mg. The average dry weight of *T. terrestris* was 2 mg.

In summary, (i) $C^{14}O_2$ was released in large quantities by respiration, and (ii) $C^{14}O_2$ was reabsorbed in some manner by the fungus cultures. Errors in translocation studies due to absorption of $C^{14}O_2$ can be minimized by using both a trap of NaOH and a directional flow of fresh air to remove atmospheric contamination.

Because of the interrelation between the mycelium and the agar medium, it was not possible to conclude definitely that the site of $C^{14}O_2$ reabsorption was fungal hyphae. The $C^{14}O_2$ could also be absorbed first by the agar medium and then diffuse into the mycelium.

Regardless of the exact site of absorption, the ultimate incorporation of C^{14} into the fungus can make the results of translocation studies misleading and should be taken into account. Although the data presented here result from the use of D-glucose-C^{14}, the use of other organic compounds labeled with C^{14} could produce similar problems.

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References and Notes

1. E. P. Hill, in *The Fungi*, G. C. Ainsworth and A. S. Sussman, Eds. (Academic Press, New York, 1965), vol. 1, pp. 457–463.
2. V. W. Cochrane, *Physiology of Fungi* (Wiley, New York, 1958), p. 228.
3. K. F. Lewis and S. Weinhouse, *J. Amer. Chem. Soc.* **73**, 2906 (1951).
4. K. H. Schutte, *New Phytol.* **55**, 164 (1956).
5. S. L. Thrower and L. B. Thrower, *Nature* **190**, 823 (1961).
6. O. Modess, *Symbolae Bot. Upsal.* **5**, 1 (1941).
7. F. N. Hayes, *Tech. Bull. No. 1* (1963), Packard Instrument Co.
8. Supported by NSF grant GB-3116.

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Collagen-Like Fragments: Excretion in Urine of Patients with Paget's Disease of Bone

Abstract. *Patients with Paget's disease of bone excrete, in the urine, polypeptides that have amino acid composition and other properties resembling those of fragments of collagen. The pattern of isotope incorporation in vivo suggests that these fragments are derived from collagen that has been synthesized and rapidly degraded, or that they are rapidly synthesized but not incorporated into tropocollagen molecules.*

The characteristic manifestation of Paget's disease is an increased rate of remodeling of bone accompanied by distortion of its architecture. Patients with this disease excrete in the urine large amounts of peptide-bound hydroxyproline which is thought to reflect the accelerated turnover of bone collagen (*1, 2*). In an attempt to understand the pathogenesis of this disease, we have isolated from the urine relatively large, collagen-related polypeptides and are studying their origin and manner of production.

Eight adult subjects with active Paget's disease of bone were maintained on gelatin-free diets; urine samples were collected and kept in the cold under toluene. The total daily excretion of hydroxyproline (*3*) in these patients ranged from 123 to 980 mg (normal adults excrete less than 40 mg per 24 hours). After exhaustive dialysis of the urine against large volumes of 0.15*M* NaCl followed by water, the nondialyzable material, amounting to approximately 10 percent of the total hydroxyproline, was lyophilized. It was then subjected to gel filtration on columns of Sephadex G-75 (125 by 3 cm), equilibrated with 5*M* LiCl and 0.01*M* tris, *p*H 7.4 (Fig. 1); a single hydroxyproline-containing zone was observed. This material was dialyzed against water, lyophilized, and then passed through smaller columns of Sephadex G-75 (110 by 1 cm) in pyridine–acetic acid buffer, *p*H 4.5. The single protein-containing peak coincided with the peak containing hydroxyproline. This same fraction, subjected to gel filtration on columns of Bio-Gel P-6 (73 by 2.5 cm), emerged with the void volume. This material was not retained by carboxymethyl cellulose at *p*H 4.8 and an ionic strength of 0.06 (*4*), and therefore was subjected to anion-exchange chromatography on columns of diethylaminoethyl- (DEAE) cellulose equilibrated with 0.05*M* tris, *p*H 8.2, with the use of a linear gradient of NaCl (Fig. 2.) The hydroxyproline-containing material was fractionated into two to four major components, each of which was then desalted by filtration on columns of Bio-Gel P-6.

Amino acid analyses (*5*) of material from comparable peaks obtained by DEAE-cellulose chromatography have thus far been performed on samples from four of the eight patients (Table 1). Amino acid composition of the urinary peptides resembled the composition of mammalian collagens in general and are shown in comparison with collagen from human bone (*6*). Ultraviolet absorption spectra revealed low absorbancy at 280 mμ compared with that at 230 mμ, which is consistent with a low content of tyrosine and tryptophan, a characteristic feature of mammalian collagens and gelatins. A 5-mg portion of one of the polypep-

tides was incubated in 0.05*M* tris, (*p*H 7.5) and 0.001*M* $CaCl_2$ at 25°C for 2 hours with 25 μg of purified clostridial collagenase (*7*). The polypeptide was degraded by the collagenase, as shown by a total increase in reactive amino groups of 3.2 μmole of leucine equivalents (*8*).

The molecular weight of these collagen-like urinary polypeptides has been estimated, on the basis of their behavior on gel filtration and from the preliminary results of high-speed sedimentation equilibrium methods (*9*), to be approximately 8000 to 9000. Optical rotatory dispersion curves of the partially purified polypeptides at 40°C in 0.001*N* HCl, over the range 350 to 195 mμ, were similar to those described for gelatin (*10*). Cooling at 9°C resulted in a rapid increase of negative rotation of approximately 40 percent at 208 mμ. One of the polypeptide fractions purified by DEAE-column chromatography showed negative optical rotation, $[\alpha]_{313}^{40} = -932°$, which slowly increased to $[\alpha]_{313}^{26} = -1206°$ during 11 days in 0.25*M* sodium citrate buffer (*p*H 3.7) at 26°C. These observations are consistent with renaturation to a collagen helix (see *11*).

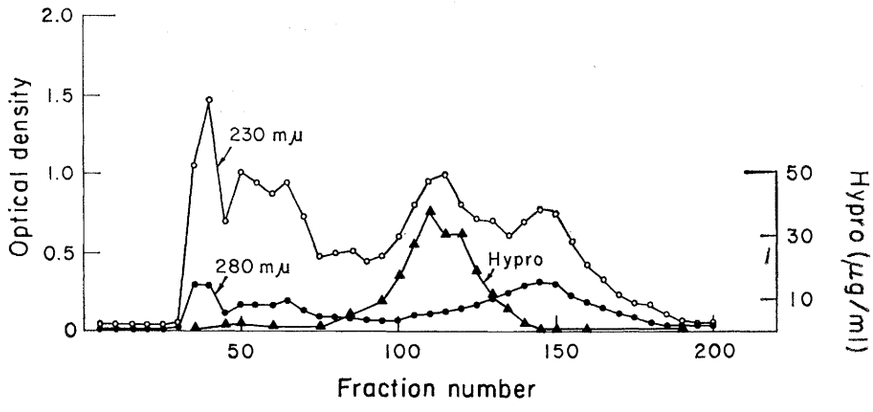

Fig. 1. Elution pattern from column of Sephadex G-75 (125 by 3 cm) of specimen of dialyzed, lyophilized urine from a patient (L.E.) with Paget's disease. The column was equilibrated, at room temperature, with 5*M* LiCl and 0.01*M* tris-HCl, *p*H 7.4; fractions were approximately 6 ml. Open circles indicate optical density (OD) at 230 mμ and closed circles OD at 280 mμ, as shown on the ordinate to the left. Triangles indicate hydroxyproline (*Hypro*) in micrograms per milliliter, as shown on the ordinate to the right.

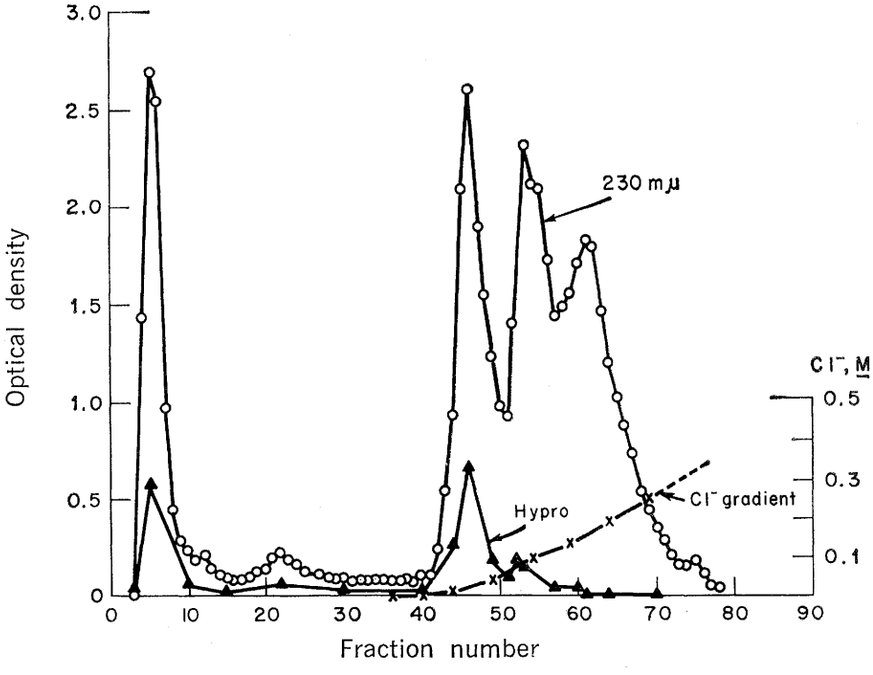

Fig. 2. Elution pattern from column of DEAE-cellulose (9 by 1 cm) of urinary polypeptide from patient (T.O.) with Paget's disease, after partial purification by gel filtration on columns of Sephadex G-75 and Bio-Gel P-6. The column was equilibrated with 0.05*M* tris-HCl, *p*H 8.2, at room temperature. At fraction 35 a gradient was begun with 1.0*M* NaCl in 0.05*M* tris-HCl, *p*H 8.2, in the reservoir; fractions of approximately 2 ml were collected. Open circles indicate optical density (OD) at 230 mμ; triangles, OD at 560 mμ for the chemical determination of hydroxyproline (*Hypro*) in aliquot portions of selected fractions, as shown on the ordinate to the left. The NaCl gradient (as Cl^-) is indicated by ×, as shown on the ordinate to the right.

It has previously been suggested from data obtained with the use of precursor ^{14}C-proline in rats that urinary hydroxyproline is derived from at least three different pools with half-lives of about 1 day, 5 days, and 50 to 100 days (*12*). In an attempt to determine whether the urinary collagen-like fragments described above are derived from pools with a short or long half-life, we initiated studies of in vivo isotope incorporation. Single doses of uniformly labeled ^{14}C-L-proline (187 and 107 μc, respectively) were administered orally to two subjects who had extensive active Paget's disease. Urine samples were collected at intervals, and portions of these samples were then dialyzed for 1 or 2 hours against equal volumes of water to obtain a dialyzable fraction containing small peptides. Material retained in the dialysis sac, which included the polypeptide collagen-like fragments, was then exhaustively dialyzed against large volumes of water to remove any remaining small hydroxyproline-peptides. The specific activity of ^{14}C-hydroxyproline (*13*) was determined on hydrolyzates of the undialyzed samples, polypeptide fractions (retained after dialysis), and small peptide fractions (dialyzable). The method used for determination of specific activity of the hydroxyproline was designed specifically for the assay of small amounts of ^{14}C-hydroxyproline in the presence of great excess of ^{14}C-proline and other radioactivity not attributable to hydroxyproline. Specific activity of ^{14}C-hydroxyproline reached a maximum in all fractions 3 to 5 hours after the administration of ^{14}C-proline, as shown for the first subject (Fig. 3). The peak specific activity (70 disintegrations per minute per micromole) in the retentate (*14*) was higher than in untreated urine (19 dpm/μmole) or in the diffusate (13 dpm/μmole). In the second patient,

the highest specific activity values were 44 dpm/μmole (retentate), 17 dpm/μmole (untreated urine), and 15 dpm/μmole (diffusate). These results were confirmed by analysis of the specific activity of fractions eluted from columns of Bio-Gel P-6, after placing on the column a sample of the undialyzed urine collected between 2 and 4 hours after administration of ^{14}C-proline. Specific activity of the ^{14}C-hydroxyproline eluted in the void volume of this column was approximately eightfold greater than that of the peptides of lower molecular weight.

Urinary hydroxyproline-containing polypeptides described in this report seem to be larger than those found in normal urine, as reported by Bourillon and Vernay (*15*). In our studies, normal urine does contain small amounts of hydroxyproline in the nondialyzable fraction, the composition of which has not yet been established. The amino acid composition of the glycopeptides containing hydroxyproline and sarcosine isolated from the urine of patients with sprue (*16*) is different from the composition of the urinary polypeptides from patients with Paget's disease. No evidence of sarcosine was found in our amino acid analyses.

Results of the isotope incorporation studies, which showed that at least some of these collagen-like fragments were rapidly and preferentially labeled and probably derived from a pool with a short half-life, suggest that they are not products of degradation of older or mature collagen. Since these hydroxyproline-polypeptides are synthesized in patients with Paget's disease at a greater rate than are other components contributing to the bulk of urinary hydroxyproline, they must originate either from collagen newly synthesized and rapidly degraded or from fragments rapidly synthesized but not incorporated into tropocollagen molecules. The size and composition of these urinary polypeptides suggest that they could be fragments of the collagen molecule of a type proposed by Gallop and his associates (*17*). It remains to be determined whether these collagen-like fragments are different from material excreted in smaller amounts in normal urine, and therefore are related to the primary defect in Paget's disease, or whether their presence in large quantity in the urine of these patients is simply a reflection of a marked increase in bone turnover.

Table 1. Amino acid composition of urinary polypeptides and human bone collagen. C.L., M.A., T.O., and L.E. are the patients. Subscript A refers to the polypeptide, from each patient, that was eluted in the first fractions of the DEAE-cellulose column; subscript B, to the peptide, from each patient, that was eluted at the beginning of the salt gradient from the DEAE-column.

Amino acids	Urinary polypeptides (amino acid residues/1000 total amino acid residues) from patients							Human bone collagen
	C.L.$_A$	M.A.$_A$	T.O.$_A$	C.L.$_B$	M.A.$_B$	L.E.$_B$	T.O.$_B$	
3-Hydroxyproline*								0.5
4-Hydroxyproline	128	144	141	106	103	112	99	98
Aspartic acid	77	59	60	93	92	88	107	48
Threonine	29	31	36	23	23	29	47	21
Serine	53	39	55	37	37	46	52	39
Glutamic acid	70	68	50	95	96	91	87	74
Proline	106	120	152	75	75	83	106	116
Glycine	313	291	265	313	305	300	233	317
Alanine	90	93	73	142	152	128	103	112
Valine	32	36	49	26	17	42	25	23
Methionine†								4
Isoleucine	3	8	9	3	2	5	9	10
Leucine	6	15	12	7	8	9	23	26
Tyrosine	2	3	2	1	1	2	4	4
Phenylalanine	3	7	5	2	3	4	8	14
Hydroxylysine	7	10	11	5	4	4	7	5
Lysine	47	31	39	43	47	23	47	30
Histidine	4	8	7	2	4	7	8	7
Arginine	27	38	26	28	32	28	20	51

* 3-Hydroxyproline was not detected in the urinary polypeptides. † Small amounts of methionine were present in the urinary polypeptides, but the peaks were too broad to permit accurate calculation.

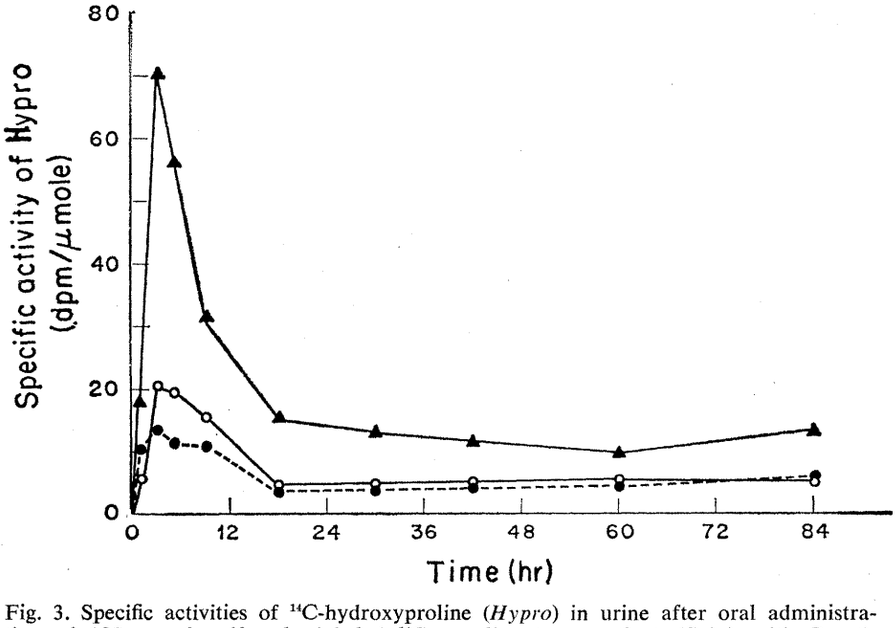

Fig. 3. Specific activities of ^{14}C-hydroxyproline (*Hypro*) in urine after oral administration of 187 μc of uniformly labeled ^{14}C-L-proline to a patient (S.A.) with Paget's disease. On the ordinate are plotted specific activities of hydrolyzates of untreated urine, material that passed the dialysis membrane (diffusate), and material retained after exhaustive dialysis of the urine (retentate). Times plotted indicate midpoints of urine-collection periods. Open circles, untreated urine; closed circles, diffusate; and triangles, retentate.

Results of the isotope incorporation studies, which showed that at least some of these collagen-like fragments were rapidly and preferentially labeled and probably derived from a pool with a short half-life, suggest that they are not products of degradation of older or mature collagen. Since these hydroxyproline-polypeptides are synthesized in patients with Paget's disease at a greater rate than are other components contributing to the bulk of urinary hydroxyproline, they must originate either from collagen newly synthesized and rapidly degraded or from fragments rapidly synthesized but not incorporated into tropocollagen molecules. The size and composition of these urinary polypeptides suggest that they could be fragments of the collagen molecule of a type proposed by Gallop and his associates (*17*). It remains to be determined whether these collagen-like fragments are different from material excreted in smaller amounts in normal urine, and therefore are related to the primary defect in Paget's disease, or whether their presence in large quantity in the urine of these patients is simply a reflection of a marked increase in bone turnover.

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References and Notes

1. T. A. Dull and P. H. Henneman, *New Engl. J. Med.* **268**, 132 (1963).
2. C. Nagant de Deuxchaisnes and S. M. Krane, *Medicine* **43**, 233 (1964).
3. D. J. Prockop and S. Udenfriend, *Anal. Biochem.* **1**, 288 (1960).
4. K. A. Piez, E. A. Eigner, M. S. Lewis, *Biochemistry* **2**, 58 (1963).
5. K. A. Piez and L. Morris, *Anal. Biochem.* **1**, 187 (1960).
6. Amino acid analyses were performed on a modified commercial model (Phoenix Precision Instruments) of the automatic amino acid analyzer by Miss Diane Brickley and Mrs. Emma Taylor of the Orthopedic Research Laboratories, Massachusetts General Hospital, under the direction of Dr. Melvin J. Glimcher. Dr. Gerald L. Mechanic assisted

in the interpretation of the chromatograms. Human cortical bone, obtained at autopsy, was extracted according to the procedure described by M. J. Glimcher and E. P. Katz [*J. Ultrastruct. Res.* **12**, 705 (1965)]. Samples analyzed were the gelatins extracted into saturated guanidine thiocyanate, *p*H 7.4, at 4°C.
7. S. Seifter, P. M. Gallop, L. Klein, E. Meilman, *J. Biol. Chem.* **234**, 285 (1959).
8. K. Satake, T. Okuyama, M. Ohashi, T. Shinoda, *J. Biochem. Tokyo* **47**, 654 (1960).
9. D. A. Yphantis, *Biochemistry* **3**, 297 (1964). Ultracentrifugation studies were performed by Dr. D. Michael Young.
10. E. R. Blout, J. P. Carver, J. Gross, *J. Amer. Chem. Soc.* **85**, 644 (1963).
11. G. Beier and J. Engel, *Biochemistry* **5**, 2744 (1966).
12. S. Lindstedt and D. J. Prockop, *J. Biol. Chem.* **236**, 1399 (1961).
13. E. C. LeRoy, E. D. Harris, Jr., A. Sjoerdsma, *Anal. Biochem.* **17**, 377 (1966).
14. E. G. Turner and J. G. Feinberg, *Nature* **184**, 1139 (1959).
15. R. Bourrillon and J. L. Vernay, *Biochim. Biophys. Acta* **117**, 319 (1966).
16. M. G. Cherian and A. N. Radhakrishnan, *ibid.* **101**, 241 (1965).
17. P. M. Gallop, *Nature* **209**, 73 (1966); O. O. Blumenfeld, M. Rojkind, P. M. Gallop, *Biochemistry* **4**, 1780 (1965); S. Seifter and P. M. Gallop, in *The Proteins*, H. Neurath, Ed. (Academic Press, New York, 1966), vol. 4, p. 153.
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Inferior Olive of the Cat: Intracellular Recording

Abstract. *The action potential evoked from inferior olivary neurons of the cat by orthodromic, antidromic, or intracellular stimulation has a large prolonged depolarization, frequently with several superimposed smaller spikes. The all-or-nothing unitary response appears to be a property of the cell, and the later spikes are not secondary to synaptic excitation by recurrent axon collaterals.*

The inferior olive (IO) complex is a major source of cerebellar climbing fibers (*1*). Eccles *et al.* (*2*) demonstrated that the Purkinje cell *D*-potential, first recorded by Granit and Phillips (*3*) with cerebellar stimulation, is the climbing fiber response (Fig. 1A) and can be evoked by either direct or reflex stimulation of IO; reflex activation presumably would be conveyed through recurrent collaterals of excitatory climbing fibers. Armstrong and Harvey (*4*) recently described the extracellular responses of IO units. Approximately one-third of their units showed an initial spike followed by several smaller spikes. They concluded, largely from recorded extracellular field potentials, that the later spikes of the unitary responses probably were excited synaptically by way of postulated IO axon collaterals (*2*, *5*). However, anatomical studies have not demonstrated such collaterals. A recent report by Sedgwick and Williams (*6*) does not mention multiple spiked responses from IO. In our investigation, intracellular recording revealed an unusual all-or-nothing potential, frequently with multiple spikes, and the evidence presented here indicates that the later spikes of the unit response are not secondary to recurrent synaptic excitation.

Cats were anesthetized with pentobarbital (intraperitoneal injection of 35 mg per kilogram of body weight), and supplements were given intravenously as required. To minimize movement, a bilateral pneumothorax was performed, and the animal was paralyzed with gallamine triethiodide. Through a ventral exposure the inferior olive nucleus was explored systematically with glass microelectrodes filled with 2.7*M* KCl or 2*M* K-citrate for intracellular recording (d-c resistance, 10 to 40 megohms in brain). In some experiments KCl electrodes filled with methyl blue dye were used for intracellular marking (*7*), and 2*M* NaCl electrodes filled with fast green FCF were used for extracellular marking (*8*). Standard intracellular recording equipment included a neutralized capacitance preamplifier and a bridge circuit for passing current through the impaling micropipette. Stimulation sites included ipsilateral pericruciate cerebral cortex (by means of bipolar silver ball electrodes), just beneath the cortical surface of anterior lobe of the cerebellum (by means of an array of concentric stainless steel electrodes), and footpad of the contralateral forepaw (by means of bipolar needle electrodes). Stimulus durations were 0.1 msec or less, usually applied as single shocks, but occasionally a train of three stimuli was used at a frequency of 1000 per second.

In ten cats, 106 units were studied by either extracellular or intracellular recording. Units were located in IO histologically by marking with dye associated with micrometer measurements and physiologically by field potentials following cerebral and cerebellar stimulation (*4*). Unit potentials from olive neurons were so unusual that identification was not a problem. Forty-six units were evoked antidromically from cerebellar stimulation; these had latencies ranging between 2.5 and 4.5 msec and followed stimulation rates of 100 to 200 per second. The latency from ipsilateral cerebral cortex (38 units) was 12.8 ± 3.6 msec (mean and standard deviation); that from contralateral forepaw (87 units) was 21.7 ± 8.2 msec. Convergence of cerebral and spinal inputs was a common finding and occurred in 31 dorsal accessory olive units and two principal olive units (ventral lamella).

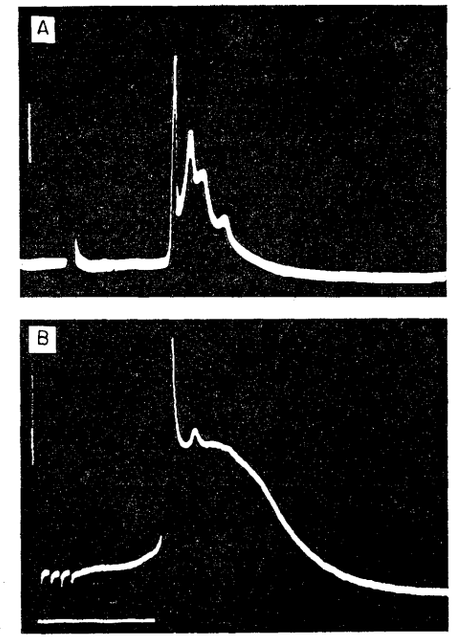

Fig. 1. (A) Response of climbing fiber recorded intracellularly from Purkinje cell. Response evoked by stimulation of inferior olive. [Reproduced with permission from Eccles *et al.* (*2*)]. (B) Intracellular action potential of an inferior olive cell evoked by stimulation of ipsilateral cerebral cortex. Top of vertical bar is zero potential and length represents 20 mv; time calibration, 10 msec.

The extracellular unitary potentials generated by the IO cells were similar to those reported by Armstrong and Harvey (*4*) and consisted of one to five initially positive diphasic or monophasic spikes (Fig. 2, I and J). The number of spikes in a unitary response was variable on successive trials, even when the strength of the stimulus was kept constant, and multiple spikes occurred more frequently with ortho-