A Critical Evaluation of the Selective Tritiation Method of Determining C-Terminal Amino Acids and Its Application to Luteinizing Hormone*

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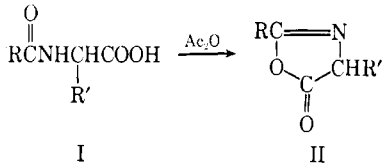
ABSTRACT: Selective tritiation has been proposed by Matsuo *et al.* (Matsuo, H., Fujimoto, Y., and Tatsuno, T. (1966), *Biochem. Biophys. Res. Commun. 52*, 69) as a method for identifying C-terminal amino acids in proteins and peptides. The C-terminal amino acids are tritiated *via* oxazalone or cyclic anhydride formation by the action of : (A) ^3H_2O and pyridine in acetic anhydride–dioxane, (B) ^3H_2O in acetic anhydride, or (C) acetic anhydride and pyridine in ^3H_2O. The present studies reveal a number of serious limitations to the use of methods A and B in determining C-terminal amino acids in proteins. Most proteins are poorly soluble in the organic solvents used in these procedures. This makes it difficult, if not impossible, to identify the C-terminal amino acids of large molecules by either of these methods. Secondly, C-terminal glutamic and aspartic acids cannot be differentiated from non-C-terminal glutamic or aspartic acid residues by method A or B. Serine and threonine cannot be identified by method A or B since they form unsaturated oxazalones under the conditions of the experiment. C-Terminal proline cannot be identified by method A because it does not form an oxazalone and its detection is complicated by tritiation of non-C-terminal glutamic and aspartic acid residues by method B. Method C, which employs an aqueous solvent, was found to be the most generally applicable for C-terminal amino acid determinations in proteins. This method was used to confirm the presence of serine and aspartic acid as C-terminal amino acids in ovine-luteinizing hormone as found by Ward *et al.* (Ward, D. N., Fujino, M., Showalter, M., and Ray, N. (1967), *Gen. Comp. Endocrinol. 8*, 289) by the hydrazinolysis and carboxypeptidase methods.

It has been reported (Matsuo *et al.*, 1965–1967) that C-terminal amino acids in polypeptides can be identified by a novel selective tritium-labeling technique. It was shown that many *N*-acetylamino acids and C-terminal amino acids of polypeptides (I) undergo cyclization in the presence of acetic anhydride to form oxazalones (II). The oxazalones contain an active hydrogen and readily incorporate tritium when treated with ^3H_2O and pyridine. Hydrolysis produces a mixture of amino acids which can be separated by paper chromatography. Matsuo's observations indicate that the C-terminal amino acid is the only one to incorporate tritium as measured by a radiochromatogram scanner.



I II

Results reported by these workers (Matsuo *et al.*, 1966) indicate that C-terminal aspartic acid and proline do not form an oxazalone and hence do not incorporate tritium under the above conditions (method A). Further experiments did, however, show that these amino acids could be tritiated under acid-catalyzed conditions (method B). This led to the proposal (Matsuo *et al.*, 1966) that the following C-terminal amino acids could be identified by the base-catalyzed procedure (method A): Gly, Ala, Val, Leu, Ile, Phe, Tyr, Try, Arg, Met, Ser, Thr, CySH, Glu, Gln, and Asn, while C-terminal Asp and Pro could be identified by the acid-catalyzed method.

The present tritiation experiments were undertaken to confirm the presence of serine and aspartic acid as C-terminal amino acids in ovine-luteinizing hormone as found by Ward *et al.* (1967) by the carboxypeptidase and hydrazinolysis methods. The studies have revealed that selective tritiation can be used as a convenient method of determining C-terminal amino acids in proteins and peptides. The method suffers from several serious limitations when the procedure is carried out in nonaqueous solvents (methods A and B) but most of the limitations can be overcome by employing ^3H_2O as the solvent for the reaction (method C).

Materials and Methods

The synthetic peptides and their *N*-carbobenzoxy derivatives were purchased from the Cyclo Chemical

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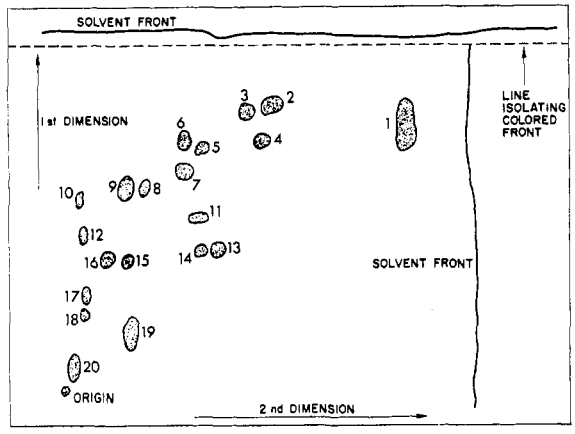


FIGURE 1: Separation of amino acids by two-dimensional thin-layer chromatography on powdered cellulose. The solvents are: first dimension: isopropyl alcohol–formic acid–water (40:2:10, v/v); second dimension: *t*-butyl alcohol–methyl ethyl ketone–0.88 M NH_3–water (50:30:10:10, v/v). 1 = DNP derivatives; 2 = leucine; 3 = isoleucine; 4 = phenylalanine; 5 = methionine; 6 = valine; 7 = tyrosine; 8 = proline; 9 = alanine; 10 = glutamic acid; 11 = threonine; 12 = aspartic acid; 13 = glucosamine; 14 = galactosamine; 15 = serine; 16 = glycine; 17 = arginine; 18 = lysine; 19 = histidine; and 20 = cystine.

Corp. Beef insulin was obtained from the Elanco Products Co. Ovine LH[1] was prepared by chromatography on carboxymethylcellulose followed by gel filtration on Sephadex G-100 (Ward *et al.*, 1967). The dinitrophenyl derivative of this protein was prepared by the sodium bicarbonate procedure of Fraenkel-Conrat *et al.* (1955). Arginine-vasopressin was generously supplied by Dr. Roger Guillemin of Baylor University College of Medicine.

C-Terminal Determinations. METHOD A. This method, which is essentially the same as reported by Matsuo *et al.* (1966), involves the base-catalyzed tritiation of oxazalones formed from C-terminal amino acids in an essentially nonaqueous system. The protein or peptide (200 nmoles) was dissolved or suspended with stirring in a mixture of 2 ml of anhydrous dioxane and 2 ml of acetic anhydride. The reaction was refluxed for 30 min and the solvents were removed under vacuum at 40°. A solution of 25 μl of ^3H_2O (25 mCi) in 1 ml of dimethylformamide and 1 drop of pyridine was added to the reaction vessel and reaction was allowed to proceed at room temperature for 1 hr. The solvents were removed under reduced pressure and the residue was dissolved in 1 ml of water which was then evaporated *in vacuo*. The residue was washed with an additional five 1-ml portions of water to assure complete removal of the washable isotope. The residue was hydrolyzed in 0.5 ml of 6 N HCl at 110° for 24 hr. This procedure is identical with that of Matsuo *et al.* (1966) except the quantities have been reduced twofold to avoid handling excessive quantities of tritium. Since our method of analysis (*vide infra*) is more sensitive than that used by Matsuo *et al.* (1966), this reduction is readily accommodated.

The hydrochloric acid was removed in a vacuum desiccator. The resulting amino acid mixture was dissolved in distilled water and the amino acids were separated by two-dimensional thin-layer chromatography according to the method of Jones and Heathcote (1966). The volume of the solution was selected so that 5 μl applied to each plate contained no more than 50 nmoles of any one amino acid to ensure good resolution by the chromatography. The amino acids were detected by spraying the thin-layer plates with 0.2% ninhydrin in 90% isopropyl alcohol–5% collidine and allowing them to stand no more than 2 hr at room temperature.

The adsorbed amino acids were scraped from the thin-layer plates along with the cellulose powder as described by Mangold (1965) and were collected by means of a vacuum pipet for transfer to a counting solution (5 g of PPO and 0.1 g of dimethyl-POPOP per l. of toluene). The vials were shaken and radioactivity was measured with a Packard Model 314EX liquid scintillation spectrometer. In some cases, the spots from two or more plates were combined in one counting vial to assure the presence of an adequate quantity of the amino acid; this was particularly important in the case of the larger proteins where the quantity of C-terminal amino acid constitutes only a small portion of the total amino acids. The number of plates combined was sufficient to bring the expected C-terminal amino acid to a molar quantity comparable to that employed in the peptide studies.

METHOD B. This procedure was used in an attempt to detect C-terminal aspartic acid which does not form an oxazalone and cannot be detected by method A. It has been reported by Matsuo *et al.* (1966) that C-terminal aspartic acid forms a cyclic anhydride which can be tritiated under acid-catalyzed conditions.

The protein or peptide (200 nmoles) was dissolved or suspended with stirring in 3 ml of acetic anhydride. ^3H_2O (50 mCi, 50 μl) was added and the reaction was refluxed for 1 hr. The solvents were removed under vacuum and the residue was washed six times with distilled water. The sample was hydrolyzed and radioactivity was measured as outlined in Method a.

METHOD C. This method, a modification of one described by Matsuo *et al.* (1966), was utilized to increase the solubility of proteins and to facilitate detection of C-terminal amino acids. Since the procedure is carried out in an aqueous medium and oxazalones are unstable under these conditions, it is assumed that tritiation occurs *via* the transient formation of an oxazalone which is in equilibrium with the uncyclized amino acid (Matsuo *et al.*, 1966).

The protein (36 nmoles) was dissolved in 0.1 ml (100 mCi) of ^3H_2O. Pyridine (0.2 ml) and acetic anhydride (0.05 ml) were added and the solution was allowed to stand at room temperature for 3 hr. The solvents were removed *in vacuo* at 40° and the residue was washed six times with distilled water. The sample was hydrolyzed and radioactivity was measured as described under method A.

[1] Abbreviations used: LH, luteinizing hormone; DNP-LH, dinitrophenylated luteinizing hormone; Arg-VP, arginine vasopressin; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; CBZ, carbobenzoxy.

TABLE I: Results of C-Terminal Determinations on Synthetic Peptides and *N*-Carbobenzoxy Peptides.

Peptide:	Gly-Leu-Tyr	Gly-Ser	Asp-Leu	CBZ-Glu-Tyr	Ala-Thr	CBZ-Gly-Asp
nmoles counted:	47	50	68	46	50	350
Method:	A	A	A	A	A	B
			Net Counts per Minute			
Ser		14				
Asp			60			2857
Tyr	445			233		
Glu				258		
Leu	7		157			
Gly	2	11				177
Ala					7	
Thr					9	

TABLE II: Results of C-Terminal Determinations on Proteins and Natural Peptide.

Protein:	LH	DNP-LH	DNP-LH	LH	Arg VP	Insulin
nmoles counted:	18	15	15	18	40	14
Method:	A	A	B	C	A	C
			Net Counts per Minute			
Asp	75	31	2638	78	0	70
Thr	0	0	81	0		1
Ser	0	9	9	80		0
Glu	28	29	2215	6	1	5
Pro	0	3	120	0	0	0
Gly	0	3	288	12	2	0
Ala	0	6	51	1		73
Cys	0	3	31	0	3	1
Val	0	8	37	0		0
Met	0	2	27	1		
Ile	0	8	34	4		0
Leu	0	9	38	0		0
Tyr	1	*a*	*a*	1	1	2
Phe	0	6	21	0	0	0
Glucosamine	0	2	6	0		
Galactosamine	0	0	8	0		0
Lys	14	*b*	*b*	9		2
His	8	*c*	*c*	10		1
Arg	0	2	27	8	0	1
DNP derivatives		12	205			

[a] Converted into *O*-DNP-tyrosine. [b] Converted into ε-DNP-lysine. [c] Converted into imidazole-DNP-histidine. Arg VP = arginine-vasopressin.

Results and Discussion

The location of the various amino acids on the thin-layer chromatograms is illustrated in Figure 1. The results obtained from C-terminal determinations on various proteins and peptides are reported in Tables I and II. It will be noted that method A gives excellent results with simple peptides such as Gly-Leu-Tyr where the C-terminal tyrosine was the only amino acid to incorporate significant levels of radioactivity (Table I).

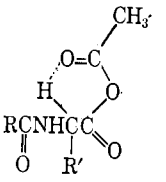
An attempt to detect the C-terminal serine of ovine LH (Ward *et al.*, 1967) by method A failed (Table II). It is significant that both the aspartic and glutamic acids of LH incorporated tritium although glutamic acid is not C-terminal (Ward *et al.*, 1967) and C-terminal aspartic acid does not form an oxazalone under these conditions (Matsuo *et al.*, 1966). In order to increase

the solubility of LH and to facilitate detection of the C-terminal serine, DNP-LH was studied. DNP-LH was found to be more soluble but the C-terminal serine again failed to incorporate significant quantities of radioactivity (Table II).

By either method A or B the DNP-LH (Table II) showed slight incorporation into several amino acids which suggests some degradation during dinitrophenylation. (The DNP-LH was isolated by acid precipitation with no further purification.) Aspartic and glutamic acids again incorporated tritium which could not be removed by washing with 1 M ammonia so it must be assumed that more than a simple hydrogen exchange at the carboxyl group is involved. Experiments carried out with dipeptides (CBZ-Glu-Tyr and Asp-Leu) confirm the observation that glutamic and aspartic acids incorporate radioactivity even if they are not C terminal (Table I).

A possible explanation for the incorporation of tritium by non-C-terminal glutamic and aspartic acid residues is that they form mixed anhydrides with acetic acid and the resulting anhydrides undergo base-catalyzed enolization. Mixed anhydrides of acylamino acids are frequently used as starting materials in the synthesis of peptides (Wieland *et al.*, 1950). They are usually prepared by the reaction of an acylamino acid or acylated peptide with an acid chloride in the presence of a tertiary amine (Greenstein and Winitz, 1961). It is, however, possible to form mixed anhydrides by the action of a symmetrical anhydride on a carboxylic acid (Emmons *et al.*, 1953). Accordingly, it is quite likely that the free carboxyls of glutamic and aspartic acids form mixed anhydrides when the protein or peptide is heated with acetic anhydride and dioxane.

The hydrogen atom attached to the α-carbon of such compounds has been found to be activated by the anhydride group (Carter and Stevens, 1941). Neuberger (1948) suggests that this could be due to the steric arrangement of the molecule which allows the carbonyl oxygen to hydrogen bond and promote ionization of the proton on the α-carbon as shown in structure III.



III

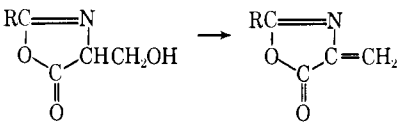
This activated α-hydrogen can be removed with a base to form a carbanion as occurs in the Perkin reaction (Buckles and Bremer, 1953). The removal of the α-hydrogen from a mixed anhydride by pyridine could then explain why non-C-terminal glutamic and aspartic acid residues incorporate tritium in this experiment.

The fact that neither the glutamine nor the aspargine of arginine-vasopressin incorporated tritium (Table II) further emphasizes that the ability of aspartic and glutamic acids to incorporate tritium is dependent on the presence of a free carboxyl group which can form a mixed anhydride. This phenomenon could be used as the basis for differentiating between aspartic acid and asparagine as well as between glutamic acid and glutamine in simple peptides since the free carboxylic acids incorporate tritium and the corresponding amides do not. It is much more convenient than electrophoretic procedures and requires very little peptide for the determination (50 nmoles). Studies are currently in progress to develop this method further.

Our failure to detect the C-terminal glycine in vasopressin is reasonable since it is amidated (du Vigneaud *et al.*, 1953). It is not surprising that aspartic and glutamic acids also incorporate high levels of radioactivity under acid-catalyzed conditions (method B) since enolization of carbonyl compounds is promoted by an acid catalyst (Bordwell, 1963).

Matsuo *et al.* (1966) report that C-terminal glutamic acid, unlike C-terminal aspartic acid, can be identified by the base-catalyzed procedure (method A) although they present no data to support this contention. It has been demonstrated (Nicolet, 1930) that heating acylated glutamic acids with acetic anhydride produces a cyclic anhydride rather than an oxazalone. The amount of tritium incorporated by a C-terminal glutamic anhydride would not be expected to be any greater than the amount incorporated by a non-C-terminal glutamic acid which had formed a mixed anhydride. This suggests that it is difficult, if not impossible, to identify a C-terminal aspartic or glutamic acid or to differentiate a C-terminal from a non-C-terminal aspartic or glutamic acid by either method A or B. A particularly good example of this difficulty is seen in the acid-catalyzed tritiation of DNP-LH (method B). Aspartic acid, which is C terminal (Ward *et al.*, 1967), cannot be differentiated from glutamic acid, which is not C terminal.

Because of our failure to detect the C-terminal serine of LH or DNP-LH, a simple peptide with a C-terminal serine was studied. As seen in Table I, the serine of Gly-Ser also failed to incorporate a significant amount of radioactivity when studied by method A. A likely explanation is that although C-terminal serine cyclizes to form an oxazalone (IV), the oxazalone rapidly dehydrates to form a more stable unsaturated oxazalone (V) (Carter, 1946). The unsaturated oxazalone contains



IV V

no active hydrogen and cannot incorporate tritium.

An analogous situation exists with C-terminal threonine which can also form an unsaturated oxazalone (Carter, 1946). Accordingly, a C-terminal amino acid determination was carried out by method A on Ala-Thr and the threonine failed to incorporate radioactivity as predicted (Table I).

Because we were unable to obtain satisfactory results from determinations carried out on proteins by method

A or B, an attempt was made to make the determinations in a more polar solvent (method C). The results in Table II show that the C-terminal asparagine and alanine of pork insulin (Sanger, 1960) and the C-terminal serine and aspartic acid of LH (Ward *et al.*, 1967) were readily identified by method C.

Aspartic acid can be identified by this base-catalyzed procedure (method C) whereas it could not be identified by the base-catalyzed procedure in an essentially nonaqueous solvent (method A). The probable reason for this is that aspartic acid cannot form a cyclic anhydride in an aqueous solvent and therefore reacts reversibly to form an oxazalone. Although the oxazalone exists only transiently, it incorporates tritium when exposed to ^3H_2O and pyridine. It appears likely that this should be the case with glutamic acid also.

Non-C-terminal glutamic acid residues in insulin and LH failed to incorporate tritium in the aqueous solvent (method C). This is almost certainly because the free carboxyl is unable to form a mixed anhydride under these conditions. The results also suggest that non-C-terminal aspartic acid residues do not incorporate radioactivity. This makes it possible to differentiate C-terminal from non-C-terminal aspartic or glutamic acids; this could not be accomplished by method A or B.

The experiments with LH also demonstrate that C-terminal serine can be identified by method C whereas it could not be accomplished by method A or B. This is seemingly because the oxazalone, which exists only transiently, is able to incorporate tritium but is unable to dehydrate to an unsaturated oxazalone in an aqueous system. One would expect that a C-terminal threonine would behave in similar fashion under the conditions of method C.

The above experiments demonstrate a number of serious limitations of methods A and B. (1) C-Terminal threonine and serine cannot be identified by method A or B because they form unsaturated oxazalones. (2) C-Terminal aspartic and glutamic acids cannot be identified by method A since they form cyclic anhydrides rather than oxazalones. Although the cyclic anhydrides do incorporate tritium under the conditions, mixed anhydrides of non-C-terminal aspartic and glutamic acids are tritiated to a similar extent. (3) C-Terminal aspartic and glutamic acids cannot be identified by the acid-catalyzed method (method B) because non-C-terminal glutamic and aspartic acid residues also incorporate high levels of tritium. (4) It is doubtful that C-terminal proline can be identified by any of these methods since it does not form an oxazalone (du Vigneaud and Meyer, 1932) and must be tritiated under acidic conditions (method B) (Matsuo *et al.*, 1966). Matsuo *et al.* (1966) identified the C-terminal proline of Phe-Arg-Try-Gly-Ser-Pro-Pro, but it should be pointed out that this peptide contains no aspartic or glutamic acid residues. According to our observations, non-C-terminal aspartic and glutamic acid residues incorporate so much radioactivity under these conditions that a C-terminal proline determination would be complicated by the incorporation of tritium into non-C-terminal residues.

These results suggest that method C is by far the most generally applicable of the three methods and is certainly the one which can be most easily applied to the identification of C-terminal amino acids in proteins and complex peptides. It does not suffer from the limitations exhibited by methods A and B and should be capable of identifying all C-terminal amino acids except proline. Another obvious advantage of method C is that most proteins are more soluble in water than in the nonpolar organic solvents used in methods A and B.

The two-dimensional thin-layer chromatographic separation of amino acids described here gives much better and more convenient resolution than the paper chromatographic procedure described by Matsuo *et al.* (1965). The sensitivity and flexibility of the assay are increased by utilizing a liquid scintillation counter rather than a radiochromatogram scanner for measuring the radioactivity incorporated by the amino acids.

There are certain anomalies in our results which have no obvious explanation, but they do deserve mention. The carbobenzoxy derivatives of the dipeptides studied showed an unusually high incorporation of tritium by the non-C-terminal amino acids by either method A (*e.g.*, compare CBZ-Glu-Tyr with Asp-Leu, Table I) or method B (*e.g.*, the Gly in CBZ-Gly-Asp, Table I). There was a rather random incorporation of tritium into several amino acids by the acid-catalyzed procedure (method B, DNP-LH, Table II). Although the data are very limited, method B gives the greatest incorporation of radioactivity but it is apparently the least specific method. Also, subsequent studies (not shown here) with method C indicate that increasing the reaction time with the tritiated water increases C-terminal amino acid incorporation of tritium, but at the expense of more random incorporation into non-C-terminal amino acids.

The basic amino acids incorporate a limited amount of tritium by any of the three procedures. This must result from protonation of the basic groups. With method C this basic incorporation is only 10% of the incorporation into the C-terminal amino acid and would only prove difficult to interpret in the instances when the protein contained a C-terminal α-carboxyl amide group (which would not be labeled) and several basic groups. For instance, with a single basic amino acid as in arginine-vasopressin (Table II) the protonation of arginine by tritium was not sufficient to be misleading.

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Reversible Dissociation of the Mouse Nerve Growth Factor Protein into Different Subunits*

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ABSTRACT: Nerve growth factor activity occurs in the crude extract of the adult male mouse submaxillary gland and can be isolated from it as a protein of approximately 140,000 mol wt. This communication describes the effects of acid and alkaline pH's on the size, net charge, biological activity, and stability of the factor protein. The large molecule is stable only between pH 5 and 8. Outside this pH range, it dissociates reversibly into smaller species with no, or considerably lower, activity. The products of the complete dissociation are three groups of subunits, one acidic (α), one basic (β), and the third intermediate in net charge (γ), all three having molecular weights of approximately 30,000. The three groups of subunits have been isolated by chromatography on CM-cellulose at low pH and each displays heterogeneity on electrophoresis. Only the β subunit elicits a nerve growth factor type of response in the standard bioassay. It accounts for 25% or less of the original activity and is markedly unstable. However, the original highly active nerve growth factor protein is spontaneously reconstituted when the three subunits are mixed at neutral pH. All three types of subunits are needed for this regeneration; α and γ subunits, while not reacting with each other, combine separately with the β subunits but the resulting complexes have the same low activity and instability as the isolated β subunit itself. Possible biological roles of the α and γ subunits are discussed.

The nerve growth factor, discovered and extensively studied by Levi-Montalcini and her colleagues (Levi-Montalcini, 1966), selectively stimulates the growth of sympathetic and embryonic sensory spinal ganglia. The factor is a protein which can be isolated from snake venom (Cohen, 1959) and the adult male mouse submaxillary gland (Cohen, 1960). The isolation of a new high molecular weight form of the nerve growth factor protein from the mouse submaxillary gland has recently been described (Varon *et al.*, 1967a). This method of isolation differs from the original procedures in using only three fractionation steps, two involving gel filtration on Sephadex and the third ion-exchange chromatography on DEAE-cellulose, and in maintaining the pH close to neutrality. The new nerve growth factor species has a molecular weight of approximately 140,000 in contrast to the lower values reported earlier (Cohen, 1959, 1960), and is obtained in considerably higher yield. Varon *et al.* (1967a) also showed that the differences in molecular weight between new and older preparations arose from the differences in the methods of isolation, in particular the use in the older method of a chromatographic separation initiated at acid pH. If the new high molecular weight form was subjected to this treatment the biological activity was found to decrease considerably and to be now

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