elephant serum enzyme we have studied in more detail[7], α-amylase, and the present group of enzymes (Table II). This is not unexpected since serum amylase is primarily of pancreatic and salivary origin.

Analysis of all the enzyme results showed that there was no variation due to the age or sex of the animals nor any due to the season or location. The serum aminotransferase levels have been reported to alter due to a variety of environmental and other factors in some breeds of healthy cattle[8–12] but not in others[13]. These include age, weight, sex, stage of lactation, season and environmental temperature. However, in our present results we found no evidence of such factors having an effect in the African elephant, although 4 individual animals did have one or more of the enzyme levels well outside the 95% probability limits. All liver function tests (including ALT levels) in these animals were normal and none of them had any other chemical evidence of disease. In the absence of further information, therefore, the causes of these high levels must remain unknown[14].

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Labile Protein-Methyl Ester: Comparison Between Chemically and Enzymatically Synthesized[1]

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Summary. The rate of hydrolysis of protein-methyl ester, the enzymatic product of S-adenosylmethionine: protein-carboxyl methyltransferase (EC.2.1.1.24) acting on oxidized ribonuclease, was measured at pH 7.1 and 8.6 at 37 °C. The half-life of the hydrolysis of the ester is 25 min at pH 7.1, and 4 min at 8.6. The rate of hydrolysis of the enzymatically formed esters at pH 7.0, in 0.1 *M* phosphate buffer, was about 25 times faster than that of esters formed chemically by reaction with methanol in HCl. The lability of the enzymatically synthesized protein-methyl ester suggests that the esterification is specific to sites such that ionization of neighboring amino acid side chains enhances the rate of the hydrolysis.

Among the various methylated amino acid residues in proteins (polypeptides) formed post-translationally by enzymatic methylation[2], the one that is formed by the methylation of carboxyl group is unique in that the product is labile in aqueous alkaline pH[3]. It has been indicated that the methyl group is linked as an ester bond at the free carboxyl groups of protein[3,4]. To support the contention further, it has been reported that the methyl-accepting capacity of substrate protein is lost after the blockage of free carboxyl groups by chemical modification[5]. Protein methylase II (S-adenosylmethionine: protein methylase II (S-adenosylmethionine: protein-carboxyl methyltransferase, EC.2.1.1.24) that methylates (esterifies) free carboxyl groups of protein substrate has been purified from various mammalian tissues[6,7]. The present communication reports the comparative rate of hydrolysis of enzymatically and chemically formed protein-methyl ester.

Materials and methods. S-Adenosyl-L-(methyl-^{14}C) methionine, 60 mCi/mmole was obtained from New England Nuclear Corporation, Boston, Mass., and ^{14}C-methanol, 58 mCi/mmole from Amersham/Searle Corporation, Arlington Heights, Illinois. γ-Aspartic and δ-glutamic acid methyl esters were obtained from Schwartz/Mann. Bovine pancreatic ribonuclease A (5 × crystallized) was obtained from Sigma, and other reagents were from local sources and of the best commercial grade available. Protein methylase II was purified from calf thymus[6] and the preparation transferred 6440 picomoles of methyl groups/min/mg protein using denatured calf thymus cytosol protein (F-P-100, ref.[3]) as substrated.

Enzymatic methylation (esterification) of oxidized ribonuclease. The general conditions for the methylation were essentially the same as those reported previously[3]. The incubation mixture contained 20 mg oxidized pancreatic ribonuclease, 0.3 ml of citrate-phosphate buffer, pH 6.0, 0.3 ml (30 μ g) of protein methylase II and 47.8 μ moles of S-adenosyl-L-(methyl-^{14}C) methionine in a final volume of 0.6 ml. The mixture was incubated at 37 °C for 1 h, at which time 23.9 μ moles of S-adenosylmethionine and 0.1 ml of protein methylase II was added again, and the incubation was continued for an additional period of 2 h. The reaction was terminated by the addition of 10 ml of cold ethanol-1 *N* HCl (39:1, v/v) and the resulting protein precipitate was removed by centrifugation. The precipitate was washed 5 times with the

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cold ethanol solution and once with anhydrous ether. The preparation contained 16,400 cpm/mg (0.29 nmol CH_3-/mg).

Chemical esterification of oxidized ribonuclease. The procedure for esterification was essentially the same as that described by CHIBNALL et al.[8]. Chemical esterification of oxidized ribonuclease with radioactive methanol was carried out in 4.5 ml of methanol containing 500 μ Ci of ^{14}C-methanol. Based on the specific radioactivity of methanol, it is calculated that the preparation contains 9.8 moles of $^{14}CH_3$-groups per mole of oxidized ribonuclease (molecular weight of 13,700 daltons). This indicates that 90% of free carboxyl groups were esterified, since there are 11 free carboxyl groups in pancreatic ribonuclease (5 aspartyl, 5 glutamyl and 1 C-terminal valine[9]).

Oxidized ribonuclease was prepared by performic acid oxidation according to published methods[5,10]. Protein concentration was estimated by the method of LOWRY et al.[11], using bovine serum albumin as the standard.

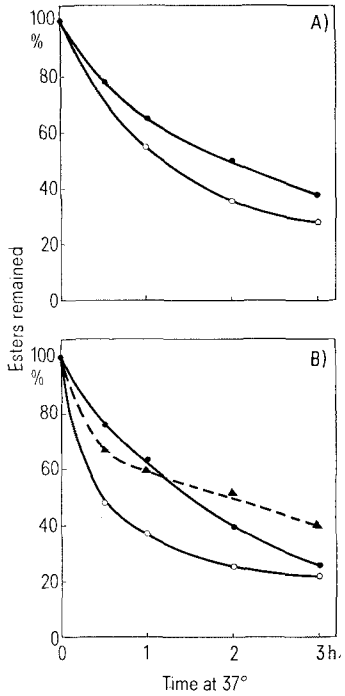

Fig. 1. A) Hydrolysis of aspartic and glutamic methyl ester at pH 8.6: 0.2 ml of 0.01 *M* γ-aspartic methyl ester or 0.05 *M* δ-glutamic methyl ester and 0.1 *M tris* buffer pH 9.2 were incubated for the indicated period of time at 37 °C. The final pH of the mixture is 8.6. At the end of the incubation, 0.2 ml aliquot of each mixture was transferred to 1 ml of alkaline hydroxylamine reagent and amount of esters were determined colorimetrically as described. Freshly prepared aspartic and glutamic methyl esters gave optical density of 0.387 and 0.265 at 540 nm respectively, and these values were taken as 100%. ●—●—●, aspartic methyl ester at pH 8.6; ○---○---○, glutamic methyl ester at pH 8.6. B) Hydrolysis of aspartic, glutamic and ribonuclease methyl ester at pH 9.7: The general experimental conditions are same as Figure 1 A except that 0.1 *M* methyl ethanolamine buffer pH 10.5 was used. The final pH of the mixture is 9.7. In the case of ribonuclease methyl ester, 0.2 ml of the solution containing 10 mg per ml was used. Optical density of 0.250 at 540 nm stands for 100% for oxidized ribonuclease methyl ester reaction. ●—●—●, aspartic methyl ester at pH 9.7; ○—○—○ glutamic methyl ester at pH 9.7 and ×---×---×, the ribonuclease methyl ester.

Results. Rates of hydrolysis of chemically prepared esters. The rates of hydrolysis of γ-aspartic and δ-glutamic acid methyl esters were determined at pH 8.6 and 9.7, and were compared with the rate of hydrolysis of chemically esterified ribonuclease at pH 9.7. As shown in Figure 1, A and B, aspartic acid methyl ester is slightly more stable than glutamic acid methyl ester at both pH's. At pH 8.6, after 1 h at 37 °C, 65% of aspartic methyl ester and 55% of glutamic methyl ester remained. Although the kinetic profile of the hydrolysis of oxidized ribonuclease methyl ester prepared chemically (Figure 1 B) is slightly different from that of amino acid ester, the initial rates of hydrolysis are comparable.

Comparison of the rates of hydrolysis of chemically and enzymatically prepared protein-methyl esters. The rates of hydrolysis of chemically and enzymatically prepared protein-methyl esters of oxidized ribonuclease are shown in Figure 2. It can be seen that the enzymatic product hydrolyzed 25 times faster than the chemical product at pH 7. At pH 8.5, less than 5% of enzymatically synthesized ester remained, while 85% of the chemically synthesized esters remained after 30 min of incubation.

Figure 3 illustrates the rate of hydrolysis of the enzymatically synthesized protein-methyl ester at pH 7.1 and pH 8.6 at 37 °C. At pH 7.1, 50% hydrolysis occurred in about 25 min, whereas at pH 8.6, this occurred in about 4 min.

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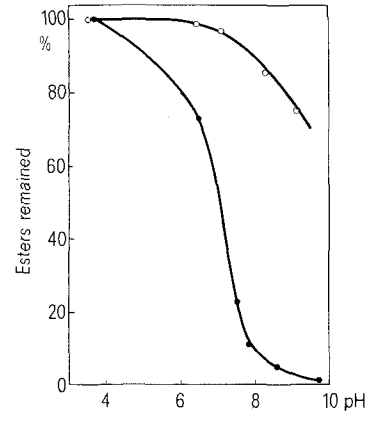

Fig. 2. The rate of hydrolysis of enzymatically and chemically esterified oxidized ribonuclease: 0.05 ml of enzymatically esterified oxidized ribonuclease (16,000 cpm, 1 mg) or 0.1 ml of chemically esterified oxidized ribonuclease (5,200 cpm, 2 mg) were added to 0.3 ml of 0.1 *M* various buffers to total volume of 0.5 ml. Final pH of the mixture was checked carefully after mixing all the reactants. Citrate-phosphate buffer for 4–6, phosphate buffer for pH 6.1–7.5, and *tris* HCl buffer for pH 7.9–10 were used. After 30 min at 37 °C, 1 ml of 30% Cl_3CCOOH was added. The precipitates were washed twice with 15% Cl_3CCOOH and once with cold ethanol. Finally the precipitates were transferred with the aid of 0.2 ml 1.5 *N* NH_4OH, to a vial containing 10 ml of Bray's solution to count remaining radioactivity. 14,000 cpm for enzymatically esterified oxidized ribonuclease and 5,200 cpm for chemically esterified oxidized ribonuclease stand for 100%. ●—●—●, enzymatically esterified oxidized ribonuclease; ○—○—○, chemically esterified oxidized ribonuclease.

Substrate capacity of chemically esterified oxidized ribonuclease

Compound	Treatment	Methyl-^{14}C incorporated (cpm)
I	None	4,130
II	Partially hydrolyzed[a]	16,450

2 mg of each substrate protein were incubated for 15 min under the standard incubation condition with purified calf thymus protein methylase II. The reaction was terminated with 15% Cl_3CCOOH and radiomethyl incorporated into protein was recovered as previously described[3].

[a] Partial hydrolysis of chemically esterified oxidized ribonuclease was carried out in 0.1 *M* sodium phosphate buffer pH 7.2 at 37 °C for 60 min.

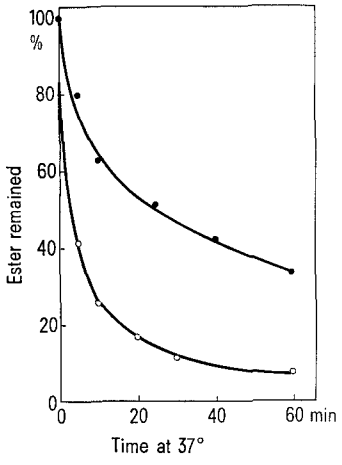

Fig. 3. The rate of hydrolysis of enzymatically esterified oxidized ribonuclease at pH 7.1 and 8.6: The general experimental conditions are the same as in Figure 2. ●—●—●, in 0.1 *M* Na phosphate pH 7.1; ○—○—○, in 0.1 *M tris* HCl pH 8.6.

Substrate capacity of chemically esterified oxidized ribonuclease. The substrate capacity of chemically esterified oxidized ribonuclease is presented in the Table. Compound I, in which 90% of free carboxyl groups was chemically esterified, was incubated at pH 7.2 and 37 °C for 60 min for partial ester hydrolysis. This mild hydrolysis is shown to remove 65% of enzymatically esterified methyl groups (Figure 3). As shown in the Table, when 4000 cpm of methyl groups were enzymatically incorporated into the compound I, 16,450 cpm were incorporated after the hydrolysis into compound II. This increase methylation is due to the selective de-esterification of the substrate protein which was previously esterified non-selectively. These results are consistent with the previous observation in which glycine-methyl ester was used to block free carboxyl groups of the same substrate[5], and thus further confirm that the free carboxyl is the site for protein methylase II, and the methyl-esters formed enzymatically at specific sites are more labile than those formed non-enzymatically as evident from Figure 1.

Discussion. Protein methylase II and 'methanol-forming enzyme' have recently been identified to be the same enzyme[6,12,13]. The enzyme transfers the methyl group of S-adenosyl-L-methionine to substrate protein. This methyl group is unstable in aqueous alkaline solution. Thus the enzymatic product can be recovered either as the protein-methyl ester or as methanol, depending on the assay method[14]. In the present report the comparison of two protein-methyl esters, prepared chemically and enzymatically, clearly shows that the enzymatic product is extremely unstable. This difference may be explained by the possibility that the enzyme modifies only specific residues in the protein and that these residues may be under the influence of the proximal amino acid side chains in a way that would cause instability of the ester bond.

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Kinetic Studies on Soluble and Membrane-Bound Dopamine β-Hydroxylase Isolated from Storage Vesicles of Heart and Adrenal Medulla of Different Species

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Summary. Identical K_m-values for soluble and membrane-bound dopamine β-hydroxylase isolated from adrenal medullary vesicles of different species were obtained; the same holds true for both forms of the enzyme of heart vesicles.

It is well known that dopamine β-hydroxylase (DBH), the enzyme which catalyzes the final step of the noradrenaline biosynthesis, is located within the catecholamine-storing vesicles. It is present in these vesicles in two forms; one of them is readily solubilized by osmotic lysis, while the other appears to be firmly membrane-bound. The distribution of both forms of the vesicular DBH seems to be species-dependent; in the chromaffin vesicles of the bovine adrenal medulla, 40–50% of DBH remains with the membranes after several washes, while in the rat, 80–85% of the DBH remain with the medullary vesicles[2]. The question arises whether these two forms of the DBH are isoenzymes or not. Gel electrophoretic studies of the membrane-bound and soluble DBH of different species showed no differences in mobility between the two forms[3]. According to HÖRTNAGL et al.[4] the amino acid composition of both forms of the bovine adrenal medullary DBH are similar; the same results were obtained by AUNIS et al.[5], while the studies of the amino acid composition of the DBH by CRAINE et al.[6] showed several significant differences. Recently, we have shown[7] that during cold exposure the activity of the soluble DBH of the vesicles of the sympathetic nerve terminals of the rat heart is increased, while simultaneous-