phile in chymotrypsin, or that, if it is, the active-site serine hydroxyl group in the initially formed sulfonyl enzyme must be somehow activated to increase its nucleophilicity. The latter conclusion appears to offer no advantage over assuming direct acylation of an activated serine hydroxyl group.

Reactivation of PMS chymotrypsin follows the steps outlined in Figure 7. The first isolable product in the desulfonylation at *p*H 2, 40°, is the enzymically inactive ester III. This product may be reactivated only if desulfonylation is carried out in dilute solution (about 0.1 mg/ml); otherwise irreversible denaturation results. When the *O,N*-acyl shift is allowed to go to completion by letting this intermediate stand at *p*H 7, an active enzyme is produced. Although dinitrophenylation of isolated samples of the ester protein led to low and variable yields of DNP-serine, the DNP-serine must have been derived from the precursor of the reactivated enzyme, since the latter contained 96% of the active sites of the original α-chymotrypsin used in the experiment. DNP-serine was not found after dinitrophenylation of isolated samples of reactivated enzyme, thus excluding the possibility that N-terminal serine residues were generated by rupture of peptide bonds.

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Studies on Rabbit Muscle Enolase. Chemical Evidence for Two Polypeptide Chains in the Active Enzyme*

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End-group analyses of rabbit muscle enolase have shown that the 85,000 molecular weight active enzyme contains two polypeptide chains. Lysine was identified as the carboxy-terminal residue in both chains by hydrazinolysis and carboxypeptidase digestion. No free amino terminus could be detected in this enzyme. After extensive digestion with pronase, however, 1.2 moles of *N*-acetylalanine per mole of enzyme was isolated, and it was concluded that *N*-acetylalanine is the amino-terminal residue in both peptide chains.

Some chemical and physical properties of rabbit muscle enolase (RME)[1] have been reported by Boser (1959), Czok and Bücher (1960), Holt and Wold (1961), and Malmström (1962). In the early work it was found that the 85,000-mw enzyme gave a single

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[1] Abbreviations used in this work: RME, rabbit muscle enolase; s-RNA, solub'e ribonucleic acid; DFP, diisopropylphosphorofluoridate.

TABLE I

THE RELEASE OF AMINO ACIDS FROM RABBIT MUSCLE ENOLASE DURING DIGESTION WITH CARBOXYPEPTIDASE A AND B

Experiment Number and Condition	Amino Acids Released	Moles/Mole Enolase[a] 3 min	15 min	30 min	60 min	90 min	120 min	150 min	180 min	360 min
1. Carboxypeptidase B (1:50) added at zero time	Lysine	0.52		1.36	1.69			1.91		
Carboxypeptidase A (1:50) added at 30 min	Alanine	0		0	0.19			0.36		
2. Carboxypeptidase A (1:10) and carboxypeptidase B (1:50) added at zero time	Lysine		0.66	1.16	1.28	1.71	1.46		1.89	
	Alanine		0.08	0.17	0.34	0.52	0.79		1.26	
3. Carboxypeptidase A (1:10) and B (1:50)	Lysine									1.89
	Alanine									1.63
4. Carboxypeptidase A (1:10) and B (1:50)	Lysine									1.92
	Alanine									1.74
5. Carboxypeptidase B (1:50)	Lysine									1.42
	Alanine									0.31
6. Carboxypeptidase A (1:10)	Lysine									0.48
	Alanine									0.36

[a] Molecular weight of RME taken as 85,000 (Holt and Wold, 1961).

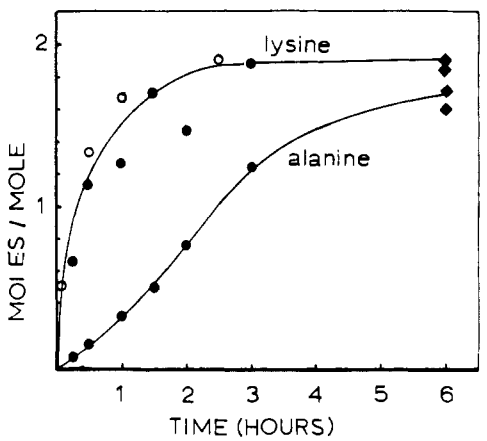

FIG. 1.—The kinetics of amino acid release from RME during digestion with carboxypeptidase A and B. The data are taken from Table I and the open circles refer to expt. 1, the closed circles to expt. 2, and the triangles to expts. 3 and 4.

peak on electrophoresis at neutral *p*H. Above *p*H 9, however, two peaks appeared, and all the activity was lost (Holt and Wold, 1961). Taking this observation as indicating the existence of subunits in RME, the present work was undertaken with the purpose of obtaining chemical evidence for multiple peptide chains in the 85,000-mw enzyme. The results of this work have given good evidence that RMA contains two peptide chains, each with a carboxy-terminal lysine residue and an amino-terminal *N*-acetylalanine.

RESULTS

The Carboxy Terminus of RME.—The release of amino acids from RME upon treatment with carboxypeptidase A and B is given in Table I and in Figure 1, and the data are consistent with an alanyl-lysine C-terminal sequence. The observed specificity of the two carboxypeptidases is in complete agreement with that reported in the literature (Neurath, 1960), and it can also be seen from experiments 4 and 5 in Table I that the specificity of the enzymes used is not an absolute one, as carboxypeptidase A slowly releases lysine and carboxypeptidase B slowly releases alanine. The two enzymes together released essentially completely 2 moles of lysine and 2 moles of alanine per 85,000 g of RME, and the fact that no other amino acids were found must mean that the next residue in the sequence was resistant to carboxypeptidase action.

The enzymatic activity of enolase was followed during the digestion in experiment 2 (Table I). The enolase remained fully active after incubation with carboxypeptidases A and B for 3 hours.

In order to establish whether the amino acids above were released from two separate alanyl-lysine sequences or from a single alanyl-alanyl-lysyl-lysine sequence, hydrazinolysis of RME was carried out. The results (Table II) from three separate experiments show

TABLE II

QUANTITATIVE ESTIMATION OF AMINO ACIDS RELEASED BY HYDRAZINOLYSIS OF RABBIT MUSCLE ENOLASE AND OF THE CARBOXYPEPTIDASE-TREATED ENZYME

	Amino Acids Released (moles/mole enolase, corrected)[a] Lysine	Alanine
Enolase[b]		
1	1.92	
2	1.95	
3	1.60	
Performic acid–oxidized carboxypeptidase A and B–treated enolase	1.25	0.20

[a] The correction factor for alanine and lysine was obtained from hydrazinolysis of a known quantity of bovine serum albumin (1 mole of carboxy-terminal alanine per 69,000 g of protein) to which had been added a known quantity of free lysine. The recovery based on this experiment was 60% for lysine and 52% for alanine in good agreement with the recoveries reported by Niu and Fraenkel-Conrat (1955). [b] Trace amounts of amino acids other than lysine were threonine 0.014, serine 0.062, glycine 0.082, and alanine 0.004 mole/mole RME. These values are uncorrected.

conclusively that there are two free carboxy-terminal lysines in the enzyme.

It was also felt of interest to establish the identity of the carboxypeptidase-resistant residue, and to this end the trichloroacetic acid–precipitated protein from experiment 3 in Table I was prepared for hydrazinolysis. This protein, having lost 1.9 moles of lysine and 1.6 moles of alanine in the carboxypeptidase treatment,

TABLE III

PAPER CHROMATOGRAPHY ON THE PRODUCTS OF HYDRAZINOLYSIS OF PRONASE FRACTION PS-1

Substance	R_F Values Ninhydrin Spray[a]	$AgNO_3$ Spray[b]	
Solvent: pyridine-aniline-water (9:1:4, v/v) (descending)			
Acetyl hydrazine		0.45	
Alanine	0.39		
Alanine hydrazide		0.64	
Hydrazinolyzate	0.39	0.45	traces (0.37, 0.30)
Solvent: collidine-α-picoline-water (5:3:2, v/v) (descending)			
Acetyl hydrazide		0.17	
Alanine	0.04		
Hydrazinolyzate	0.04	0.16	trace (0.23)

[a] Sigma commercial ninhydrin spray. [b] Ammoniacal silver nitrate solution (0.1 N $AgNO_3$–5 M NH_4OH–ethanol mixture, 1:1:2, v/v).

should contain about 1.5 moles of the resistant residue, 0.1 mole of lysine, and 0.3–0.4 mole of alanine in the carboxy-terminal position. Since cysteine is completely destroyed by hydrazinolysis, while cysteic acid is stable (Locker, 1954), the protein was oxidized with performic acid prior to hydrazinolysis. Rather surprisingly, the major free amino acid found in this experiment (Table II) was lysine (1.25 moles per mole of RME). In addition a small amount of alanine was also released, as should be expected since 0.4 mole of carboxy-terminal alanine still remained after the carboxypeptidase digestion.

From these results it was concluded that RME consists of two peptide chains, and that the carboxy-terminal sequence of each chain must be -x-lys-ala-lys. Whatever the nature of x is, it induces high carboxypeptidase resistance to the x-lysine peptide bond. As it has been reported by Smith (1954) that the sequence pro-x is resistant to carboxypeptidase action, it is possible that the fourth residue in from the carboxy terminus is a proline residue.

The Amino Terminus of RME.—Both leucine aminopeptidase and fluorodinitrobenzene failed to give any evidence of a free amino-terminal residue, suggesting that RME might be an *N*-acylated enzyme. To investigate this possibility, two separate samples of enolase (labeled PS-1 and PS-2) were digested with pronase, and the ninhydrin-negative fractions which were not retarded on Dowex-50 H^+ were collected. These fractions were subjected to paper chromatography, to hydrazinolysis followed by paper chromatography, and finally to hydrolysis and quantitative estimation of the amino acid components. The results, given in Tables III, IV and V, led to the conclusion that RME contains 2 moles of *N*-acetylalanine per 85,000 g of protein.

The only complication in arriving at this conclusion was the presence of pyroglutamic acid in the ninhydrin-negative fractions analyzed (Table IV). It was assumed, however, that this pyroglutamic acid was formed during the pronase digestion and the subsequent treatment of the digest, rather than arising from an existing pyroglutamyl amino terminus. This assumption has reasonable foundations in the fact that pyroglutamic acid is readily produced from glutamine under mild conditions (*p*H 2–10, 100°, 1 hour) (Archibald, 1945) and indeed has been found after pronase digestion of other proteins (e.g., ovalbumin, Narita and Ishii, 1962). When bovine serum albumin was digested with pronase in an experiment parallel to the enolase digestion, a significant quantity of pyroglutamic acid could be identified in the ninhydrin-negative fraction of this protein also (Tables III and V).

TABLE IV

PAPER CHROMATOGRAPHY OF THE NINHYDRIN-NEGATIVE FRACTIONS FROM PRONASE DIGESTIONS[a]

Substance	R_F Values Descending	Ascending
Solvent: butanol-acetic acid–water (4:1:1, v/v)		
N-Acetylalanine	0.81	0.76
Pyroglutamic acid	0.58	0.585
Fractions of PS-2	0.805, 0.58	0.76, 0.58 (other slight traces)
Fraction of bovine serum albumin		0.583, trace 0.435
Solvent: 1-propanol-ammonia-water (50:40:10, v/v)		
N-Acetylalanine	0.67	0.68
Pyroglutamic acid	0.56	0.59
PS-2	0.67, 0.56	0.69, 0.58
Bovine serum albumin	0.57	

[a] Detecting reagents were bromcresol green indicator spray and chlorine-iodine-starch spray for peptide bonds (Roydon and Smith, 1952).

TABLE V

AMINO ACID ANALYSIS OF THE NINHYDRIN-NEGATIVE FRACTIONS FROM PRONASE DIGESTIONS

Sample	Hydrolysis (moles/mole protein)		Hydrazinolysis (moles/mole protein)
PS-1	Alanine	0.93	Alanine 1.13[a]
	Glutamic acid	0.35	
PS-2	Alanine	1.23	
	Glutamic acid	0.50	
Bovine serum albumin	Glutamic acid	0.25	

[a] Uncorrected value. The product from the hydrazinolysis was lyophilized and applied to the analyzer column without removing the hydrazides.

The major component of the ninhydrin-negative fraction was identified as *N*-acetylalanine, and amino acid analysis of this fraction from the two separate experiments gave 0.9 and 1.23 moles of alanine per mole of digested enolase. Since no alanine hydrazide could be detected and 1.13 moles of free alanine was found after hydrazinolysis it seems reasonable to conclude that all the alanine in the fraction was present as *N*-acetylalanine. Furthermore, since the recovery of *N*-acetylalanine from the pronase-digestion mixture could not be expected to be 100%, it was concluded that there are 2 moles of *N*-acetylalanine per mole of RME, again accounting for the amino terminals of two separate polypeptide chains.

DISCUSSION

The results presented above show that rabbit muscle enolase is made up of two polypeptide chains. There is good evidence that the 85,000-mw two-chain species is indeed the active form of the enzyme and physical evidence for the reversible dissociation of the chains is also available (Winstead and Wold, 1964). Beyond the apparent identity of the amino terminals and the three carboxy-terminal residues in the two chains, there is yet no basis to claim that the chains are identical.

TABLE VI
LIST OF α-N-ACETYLATED PROTEINS

Protein	Amino-terminal N-Acetyl-amino Acid	Reference
Beef- and horse-heart cytochrome C	Glycine	Titani *et al.* (1962)
Tobacco mosaic virus protein	Serine	Narita (1958)
Turnip yellow mosaic virus protein	Methionine	Harris and Hindley (1961)
Cucumber virus protein	Alanine	Narita (1959)
α-Melanocyte-stimulating hormone	Serine	Harris (1959)
Beef fibrinogen (peptide B of cofibrin)	Threonine	Folk and Gladner (1960)
Ovalbumin	Glycine	Narita and Ishii (1962)
Chicken globin	Valine	Satake *et al.* (1963)
Calf thymus histones		Phillips (1963)
Rabbit muscle enolase	Alanine	

A list of known acetylated proteins is given in Table VI and may imply a quite general phenomenon in all species. In fact, it does not seem unreasonable to propose that all proteins are synthesized with an acetylated amino terminus. This would presumably require a separate genetic code for either acetate or for *N*-acetylamino acids. It should be mentioned here that an attempt to demonstrate acetate activation and the formation of s-RNA-acetate using yeast s-RNA and activating enzymes[2] gave negative results, and the possibility that acetate participates per se in protein synthesis in yeast thus appears unlikely. Perlman and Bloch (1963) have recently demonstrated the formation of s-RNA-acetyltyrosine, however, and so it is possible that a code for acetylamino acids does exist. The very interesting possible significance of acetylamino acids in providing obligatory interruptions in the polypeptide assembled on a single messenger which contains the coded information for several individual proteins has been discussed by Perlman and Bloch (1963).

EXPERIMENTAL

Preparation of Rabbit Muscle Enolase.—Recrystallized enolase was prepared from frozen mature rabbit muscle (obtained from Pel-Freez Biologicals, Inc.) by a modification of the procedure of Holt and Wold (1961). Protein determinations were made by the biuret-phenol method of Sutherland *et al.* (1949) and by optical density measurement. The absorbancy at 280 mμ of the recrystallized enzyme was found to be 0.9 for a 1 mg/ml solution (Holt and Wold, 1961). Enzyme activity was measured in imidazole buffer according to the published procedure (Holt and Wold, 1961).

1. *Studies on the Carboxy Terminus.*—Hydrolysis by Carboxypeptidases A and B.—Carboxypeptidase A DFP (3-times-crystallized water suspension) and carboxypeptidase B (frozen solution) were obtained from Worthington Biochemical Corp. The molar concentration of carboxypeptidase A was calculated from its absorbance at 280 mμ ($A_{280}/2.3$ = mg of carboxypeptidase per ml), assuming a molecular weight of 34,000 (Neurath, 1955). The concentration of carboxypeptidase B was calculated from its absorbance at 278 mμ (the absorbancy index at 278 mμ of a 1% solution was found to be 21.4 and the molar absorbance index is 7.35×10^4 based on a molecular weight of 34,300 [Folk *et al.*, 1960]). The rabbit muscle enolase was prepared for carboxypeptidase reaction by dialyzing against 0.05 M $KHCO_3$, *p*H 7.6, for 5 hours with a protein concentration of 15–20 mg/ml. Carboxypeptidase A was dissolved in 10% LiCl to give approximately 3.5 mg/ml. Carboxypeptidase B was dissolved in 0.05 M $KHCO_3$ buffer, *p*H 7.6 (1 mg/ml), treated with DFP (0.03 ml of a solution of 0.1% DFP in propylene glycol per ml of carboxypeptidase B solution), and incubated for 1 hour. Carboxypeptidase A and carboxypeptidase B were added to the enolase solution at a molar ratio of 1:10 and 1:50, respectively. The hydrolysis was carried out at 25° and aliquots containing 15–25 mg of protein were removed at various times. A sample was removed at zero time for a control. The reaction was stopped by precipitation by 1 volume of 10% trichloroacetic acid. The protein was centrifuged down and the supernatant was lyophilized three times to remove most of the trichloroacetic acid. The resulting samples were analyzed for amino acids.

Performic Acid Oxidization.—This was performed by the method of Hirs (1956) at −5°.

Hydrazinolysis.—A procedure similar to that of Niu and Fraenkel-Conrat (1955) was used. Samples of 0.2–0.4 μmole of enolase or the equivalent quantities of ninhydrin-negative fraction from pronase digestion (see below) were dried at 80° under vacuum. To these were added 0.5 ml of anhydrous hydrazine (95% + obtained from Matheson, Coleman, and Bell) and the samples were frozen, evacuated, and sealed. The samples were heated from 6–10 hours at 100°. The excess hydrazine was removed in a vacuum desiccator over sulfuric acid and the residue was dissolved in 1.0 ml of water to which was added 0.3 ml of benzaldehyde. The mixture was shaken at 25° for 2 hours. The benzaldehyde layer containing the condensation product of the hydrazides with benzaldehyde was removed by centrifugation. The aqueous layer was further extracted with 0.2 ml of benzaldehyde followed by 0.2 ml of ether. The aqueous samples were then dried by lyophilization and analyzed for free amino acids.

2. *Studies on the Amino Terminus.*—The Fluorodinitrobenzene Method (Fraenkel-Conrat *et al.*, 1955). —This method was used in attempts to find a free amino terminus in RME. In order to eliminate possible destruction of DNP-amino acids during hydrolysis all the possible hydrolysis variations (Porter, 1957) were employed.

The method of Narita (1958) was used in the search for an acetylated amino terminus.

Pronase Digestion.—Pronase digestion was carried out by the method of Titani *et al.* (1962). Approximately 200 mg of crystalline enolase was dialyzed against glass-distilled water for 20 hours at 4°. Final volume was 20 ml to give a concentration of 20 mg/ml. Prior to digestion the protein was denatured by varying the *p*H from 5 to 10 with 0.1 M HCl and 0.1 M NaOH, and the final *p*H was adjusted to 7.6. (The enolase which has been dialyzed against water is very readily denatured and precipitated by slight alterations in *p*H.) Two mg of pronase (*Streptomyces griseus* protease, Grade B, obtained from California Corp. for Biochemical Research) was dissolved in 1 ml of 0.01 M $CaCl_2$ and added to the enolase suspension at 35°. The cloudiness rapidly disappeared. The *p*H was maintained between 7 and 8 by additions of 0.1 M NaOH. The digestion was continued for 20 hours, and stopped by freezing and lyophilization.

The extent of hydrolysis by pronase under these

[2] We gratefully acknowledge the generous help and advice from Dr. John M. Clark, Jr., in these experiments.

conditions was determined by amino acid analysis of the digestion mixture. It was found that 40% of the protein could be accounted for as free amino acids. All the methionine, leucine, tyrosine, and phenylalanine had been released, whereas no free asparatic acid, proline, or cysteine could be detected.

Separation of the Ninhydrin-negative Fraction.—The lyophilized sample was dissolved in 3 ml of water, the *p*H was adjusted to 4 with 1 M HCl, and undissolved material was removed by centrifugation. The digest was placed on a column of purified Dowex-50-X2 (AG 50 w-X2, Bio-Rad Laboratories, hydrogen form, 200–400 mesh, 1.7 × 15 cm) and washed with glass-distilled water. The eluate fractions (25 ml each) were concentrated to a small volume on a flash evaporator. Aliquots of the concentrated solution were spotted on paper and tested with ninhydrin spray reagent (Sigma) and were also subjected to quantitative ninhydrin test (Moore and Stein, 1954). Approximately 125–200 ml was collected before any ninhydrin-positive material appeared. The ninhydrin-negative fractions were pooled, and samples were removed for hydrolysis (20 hours at 110° with 6 N HCl in sealed evacuated tubes) and hydrazinolysis (*vide supra*).

Identification of Amino Acids and Hydrazides.—This was accomplished by paper chromatography. Control samples of acetyl hydrazide and alanine hydrazide were prepared by hydrazinolysis of *N*-acetylalanine and alanylleucine, respectively. Solvent systems and color reagents are given in the tables. Quantitative amino acid analyses were performed with the Beckman/Spinco amino acid analyzer according to the method described by Moore *et al.* (1958).

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Glycine Transport by Hemolyzed and Restored Pigeon Red Cells*

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Pigeon red cells can be lysed and restored by a modification of the method used for mammalian red cells. During lysis, glycine is equally distributed, and K^+ nearly so, between cell and lysing-fluid phases. Such preparations retain glycine and K^+ at 0–7° but lose about 32% of the K^+ in 40 minutes at 39°. The lysed and restored cells can build up glycine concentration gradients only if a Na^+ gradient exists across the cell membrane. If the normal gradient is reversed (Na^+ inside, K^+ outside) lysed and restored cells pump glycine out to an extent comparable to the accumulation occurring with a normal gradient. The Na^+ dependence is discussed in relation to Christensen's hypothesis that the energy for active transport of glycine arises from a cation gradient. This hypothesis is supported by the data, although others are not excluded.

Hypotonic hemolysis of mammalian red cells is a "reversible" process in that much of the impermeability of the cell membrane, lost during hemolysis, can be restored by restoring the ionic strength and then holding at 37° (Hoffman *et al.*, 1960; Whittam, 1962). Mammalian erythrocytes lysed and restored in this way can transport Na^+ actively (Hoffman, 1962). By use of this phenomenon the composition of the red-cell interior can be varied within wide limits.

Since mammalian red cells concentrate glycine very poorly in contrast to avian cells (Christensen *et al.*, 1952a) the technique of lysis and restoration was adapted to pigeon red cells. The procedure, some

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