

## CHARACTERIZATION OF ERYTHROCYTE ESTERASES ON ELECTROPHORETIC GELS<sup>1</sup>

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### ABSTRACT

Polyacrylamide gel electrophoresis revealed a complex array of esterases in hypotonic washings and membrane fractions from rabbit erythrocytes prepared in the presence of Triton X-100. By the use of on-gel techniques, these esterases were characterized on the basis of substrate specificities, susceptibilities to inhibitors, and sensitivities to urea and heat. In addition to acetylcholinesterase, known to be present in erythrocyte membranes, the classes of enzymes were shown to be heteromorphic and examples were found for carboxylesterases, arylesterases, acylesterases and cholinester hydrolases. These data establish electrophoretic patterns of rabbit erythrocyte esterases that may serve as standards to which enzymes from physiologically altered test animals might be compared.

### INTRODUCTION

Enzymes distinguished qualitatively by gel electrophoretic techniques are useful as biological indicators of changes in composition of edible substances during preharvest, harvest, storage, and processing (Manwell and Baker 1970; Cherry 1977, 1978; Cherry et al. 1978). Changes include deletion of some enzymes, intensification of others, and/or production of new components as evidenced by quantitative and qualitative changes in bands appearing on electrophoretic gels. Enzymes in erythrocyte membrane and cytoplasm fractions may be useful in elucidating physiological changes due to nutritional imbalances in diets. Gel electrophoretic tests of enzymes in blood and other tissues are used to indicate the existence of certain disease-related physiological disorders (Wilkinson 1976; Ray and Cherry 1977). However, enzyme multiplicity that results from genetic variability, tissue ontogeny, and method of preparation (Cherry 1977, 1978) can complicate gel electrophoretic analysis. Thus, care should be taken to insure that known gel patterns

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of enzymes are developed as standards for the materials to be examined prior to evaluation of treatment effects.

Previous workers (Markert and Hunter 1959; Augustinsson 1961; Holmes and Masters 1967, 1968) showed by non-gel-electrophoretic techniques that esterase activity in mammalian tissues is due to multiple enzyme forms with widely differing substrate specificities, susceptibilities to inhibitors, pH optima, and sensitivities to urea and heat. Esterases that have been observed by quantitative methods in tissues include carboxylesterases (E. C. 3.1.1.1), arylesterases (E. C. 3.1.1.2), acylesterases (E. C. 3.1.1.6), acetylcholine hydrolases (E.C. 3.1.1.7), and cholinester hydrolases (E. C. 3.1.1.8). Erythrocytes of some mammalian species contain, in addition to the well-known membrane-bound acetylcholinesterases, other esterases that are relatively nonspecific, including arylesterases (Augustinsson et al. 1973). The present study was undertaken to demonstrate how the diversity of esterases present in rabbit erythrocytes can be standardized, using polyacrylamide disc gel electrophoretic techniques.

#### MATERIALS AND METHODS

##### *Extraction of Erythrocyte Constituents*

Blood from New Zealand white rabbits, 8 to 12 months old and weighing approximately 2500 g, was collected by cardiac puncture (approximately 45 ml per rabbit) in the presence of an anticoagulant acid-citrate-dextrose solution. Procedures for hemolyzing and washing the erythrocytes and preparing membrane ghosts were those of Cherry and Prescott (1974); membrane constituents were solubilized with 2.5% Triton X-100<sup>2</sup> in the presence of 1 mM EDTA, with or without 10 mM dithiothreitol (DTT).

##### *Gel Electrophoresis and Detection of Esterases*

Electrophoretic separation of these components was by a "standard" gel electrophoretic technique (Cherry and Prescott 1974). The identities of esterase bands on the electrophoretic gels were determined by direct on-gel staining techniques. Nonspecific esterases (general staining procedure for esterase activity) were detected by incubating duplicate gels in a mixture of  $\alpha$ - and  $\beta$ -naphthyl acetate at room temperature for 30-60 min (Cherry and Katterman 1971). The substrate mixture contained 100 ml sodium phosphate (0.1 M, pH 6.1), 5 ml 1-propanol, 30 mg fast blue salt, 1.5 ml  $\alpha$ -naphthyl acetate stock solution, and 1 ml

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<sup>2</sup>Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

$\beta$ -naphthyl acetate stock solution; the stock solutions contained 1 g of the respective naphthyl acetate in 100 ml of 50% acetone.

Acetylcholinesterase and butyrylcholinesterase activities were tested by modification of the method of Shafai and Cortner (1971). To 13 ml of sodium phosphate (0.5 M, pH 6.1) 50 mg acetylthiocholine iodide (for acetylcholinesterase) or 50 mg butyrylthiocholine iodide (for butyrylcholinesterase), 1 ml sodium citrate (1 M), 4 ml copper sulfate (0.15 M), and 2 ml potassium ferricyanide (0.05 M) were added in that order and mixed thoroughly; the gels were placed in this solution and kept at room temperature for 30-60 min.

Other substrates utilized to determine the specificities of individual esterase bands included  $\alpha$ -naphthyl butyrate,  $\beta$ -naphthyl laurate, indoxyl acetate, thiophenyl acetate, and leucyl- $\beta$ -naphthylamide. These preparations were made as stock solutions each containing 1 g of substrate per 100 ml 50% acetone, and fast blue salt was used as above (Cherry and Katterman 1971) to make the enzyme bands visible. Insoluble substrates were made as suspensions by subjecting the mixture to sonication.

#### *Esterase Inhibition*

Inhibitors of esterase activity tested in this study were diisopropylphosphorofluoridate (DPF), tri-*o*-tolyl phosphate (T-*o*-TP), phenylmethanesulfonylfluoride (PMSF), *p*-chloromercuribenzenesulfonic acid (CMBSA), eserine (physostigmine), mercuric chloride, and acetazolamide. The concentrations of inhibitors used for each experiment ranged from 0.01 to 1 mM, with each sample being subjected to the inhibitor for 15-20 min both before and after electrophoresis.

#### *Heat Sensitivity*

Sensitivity of esterases to heat was determined by incubating the electrophoretic gels in phosphate buffer (0.1 M, pH 6.1) at 55 C, and the effects of urea were investigated by soaking the gels in a solution of 10 M urea at room temperature. Both stability experiments were conducted for varying periods up to 40 min, after which the gels were equilibrated at room temperature in phosphate buffer, then incubated with the substrate that included fast blue salt for staining the enzyme bands.

### RESULTS AND DISCUSSION

Electrophoretic gels of both the hypotonic washings and the membranes of rabbit erythrocytes showed multiple, discrete bands of non-specific esterases (Fig. 1). Region 1.5 - 6.5 cm of the gel patterns revealed that several of the same enzyme bands were present both in the membranes (gels A-D) and in the washings (gels G-J). The membrane fraction, however, contained esterase activity in regions 0.5 - 1.5 cm and

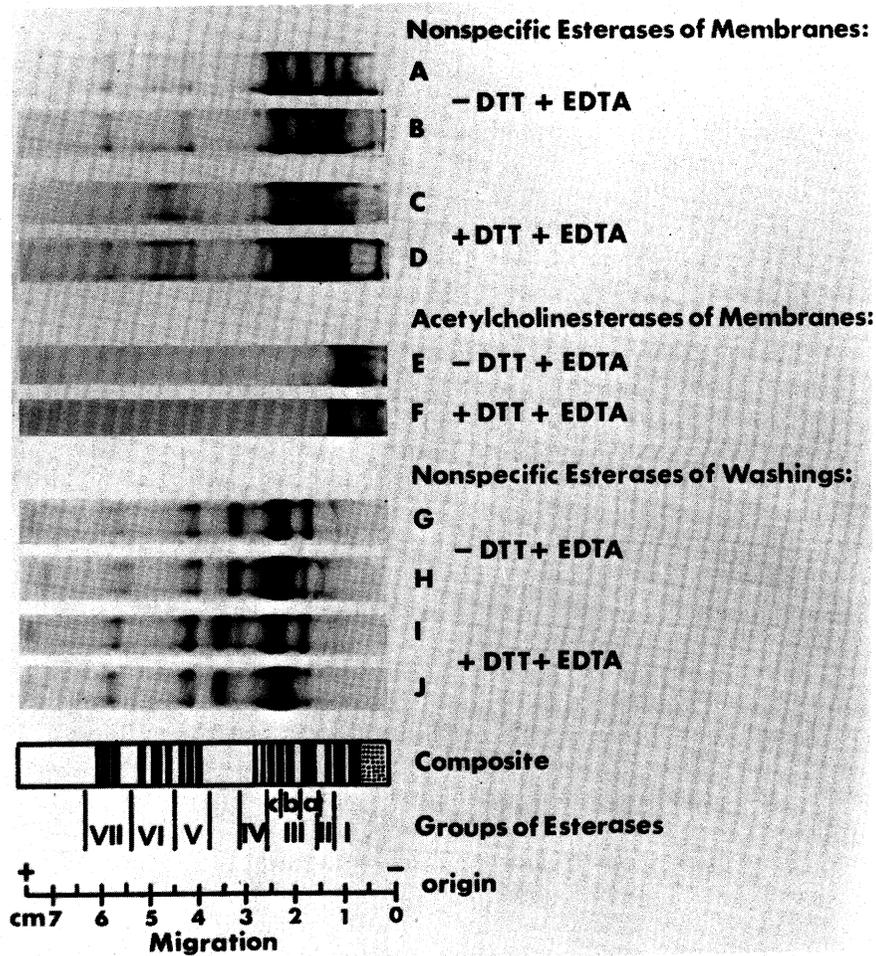


FIGURE 1. Standard polyacrylamide gel electrophoretic patterns of esterase activity in membranes and washings of rabbit erythrocytes. Esterases were solubilized in the presence of 2.5% Triton X-100 and 1 mM EDTA without (gels A, B, E, G, and H) or with 10 mM DTT (gels C, D, F, I, and J). Gels E and F were stained specifically for acetylcholinesterases, and the others were stained for nonspecific esterase activity with a mixture of  $\alpha$ - and  $\beta$ -naphthyl acetate as the substrate solution. Gels A, C, G and I, and B, D, H and J distinguish the esterases of two groups of rabbits; E and F were the same for both groups. A composite drawing showing the classes of esterase activity (I, acetylcholinesterases; II, acylesterases; III, a, b, and c, IV, and VI, carboxylesterases; V and VII, arylesterases). The bands in region 3.0 - 3.5 cm of gels G - J are hemoglobin.

4.5 - 5.5 cm, a result not clearly evident in the washings. Acetylcholinesterase activity (gels E-F; region 0 - 1.3 cm) was found only in the membrane fractions, as would be expected in light of previous reports showing that this enzyme is membrane-bound (Shafai and Cortner

1971; Wright and Plummer 1972; Ciliv and Ozand 1972; Srinivasan et al. 1972; Wheeler et al. 1972; Augustinsson et al. 1973).

Membranes and washings showed evidence of enzyme polymorphism in region 2.0 - 2.5 cm, possibly resulting from genetic variability among the rabbits (cf. gels A, C, G, and I to B, D, H, and J which distinguish two groups of rabbits). In addition, the presence of dithiothreitol (DTT) affected the number and intensity of bands in some of the samples (Fig. 1). These data indicate that the addition of reducing agents to samples, as well as genetic variability in tissues, can affect electrophoretic patterns (Carter 1973; Cherry and Ory 1973), which therefore should be interpreted cautiously, by careful comparison with adequate controls.

The results of testing the gels against various esterase substrates and with different inhibitors of esterase activity in membrane and wash fractions are presented in Table 1; the enzymes are grouped (I-VII) according to the mobilities shown in the diagram in Figure 1. The members of Group I, the slowest migrating enzymes, hydrolyze acetylthiocholine, butyrylthiocholine, thiophenyl acetate, and indoxyl acetate, in addition to the two substrates for nonspecific esterases (namely,  $\alpha$ - and  $\beta$ -naphthyl acetate). This group of enzymes is inhibited by DPF, PMSF, and eserine but not by mercuric chloride or CMBSA, and it seems to be composed largely of acetylcholinesterases. Group II consists of esterases that did not hydrolyze acetylthiocholine or butyrylthiocholine and were not inhibited markedly by any of the inhibitors tested. The enzymes in Groups III (a, b, c), IV, and VI, consisting of slow, intermediate, and rapidly migrating carboxylesterases, respectively, were inhibited partially or completely by DPF and PMSF but not by mercurials or T-o-TP. Only Groups I and VI were inhibited by eserine. Groups V and VII showed specificity typical of arylesterases, being inhibited partially by DPF, PMSF, and the mercurial reagents. Acetazolamide did not alter the activity of any esterases distinguished in the gel patterns; thus, it seems that none of these enzymes was a carbonic anhydrase.

All of the esterases (Groups I-VII) showed some degree of activity against  $\alpha$ -naphthyl acetate,  $\beta$ -naphthylacetate, and indoxyl acetate, but none showed amidase activity toward leucyl- $\beta$ -naphthylamide. Enzymes tentatively identified as acetylcholinesterases (I) showed specificity toward acetylthiocholine iodide and thiophenyl acetate, and all of the suggested carboxylesterases (III a, b, c, IV, and VI) were highly active toward  $\alpha$ -naphthyl butyrate. Similar to the acetylcholinesterases (I), the carboxylesterases with intermediate mobilities in the gels (IV) showed activity toward butyrylthiocholine iodide, a substrate that is specifically hydrolyzed by butyrylcholinesterases. Bands in Region II

TABLE I. Characterization of esterases from rabbit erythrocyte washings and membranes according to substrate specificity and susceptibility to inhibition and inactivation.

Treatment	Activity of Esterases of Group <sup>a</sup>							
	I	II	IIIa,c	IIIb	IV	V	VI	VII
<i>Substrates</i>								
$\alpha$ -Naphthyl acetate	++++	++++	++++	++++	++++	+++	++++	+++
$\beta$ -Naphthyl acetate	+++	+++	+++	+++	+	+	++	+
$\alpha$ -Naphthyl butyrate	-	++	++++	++++	++++	-	++++	-
$\beta$ -Naphthyl laurate	+	++	-	-	-	-	-	-
Indoxyl acetate	+++	++	++++	++++	+	++	+	++
Acetylthiocholine iodide	+++++	-	-	-	-	-	-	-
Butyrylthiocholine iodide	+++	-	-	-	++	-	-	-
Thiophenyl acetate	++++	+	+++	++	+	-	-	-
L-Leucyl- $\beta$ -naphthylamide	-	-	-	-	-	-	-	-
<i>Inhibitors<sup>b</sup></i>								
DPF	+	++++	+	-	-	+	-	++
T-o-TP	++++	++++	+++	+++	+++	++++	++++	++++
PMSF	+	++++	+	-	-	+	-	++
Eserine	+	++++	++++	++++	++++	++++	-	++++
CMBSA	++++	++++	++++	++++	++++	-	++++	-
Mercuric chloride	+++	+++	+++	+++	++	+	++	+
Acetazolamide	++++	++++	++++	++++	++++	++++	++++	++++
<i>Minutes of exposure to 55 C</i>								
0	++++	++++	++++	++++	++++	++++	++++	++++
3-7	++++	+++	+++	+	++	++	+++	++
12-18	+++	++	++	-	+	+	++	+
25-32	++	+	+	-	-	-	+	-
40	++	-	+	-	-	-	-	-
<i>Minutes of exposure to 10 M urea</i>								
3-7	++	++	++++	++++	++	++	++	++
12-18	+	+	+++	+++	+	-	+	-
25-32	-	-	++	++	-	-	-	-
40	-	-	+	+	-	-	-	-

<sup>a</sup>Groups I-VII denote bands with substrate specificities typical of acetylcholinesterases (I), acylesterases (II), carboxylesterases (III a, b, and c, IV, and VI), and arylesterases (V and VII). Nonspecific esterase activity of each group, as determined with a mixture of  $\alpha$ - and  $\beta$ -naphthyl acetate, is denoted by ++++; - indicates no detectable activity; + denotes trace activity; ++, +++ are intermediate amounts of activity; and +++++ is activity exceeding that observed toward the mixture of  $\alpha$ - and  $\beta$ -naphthyl acetate substrates. The latter mixture was used also as the substrate to test the effects of inhibitors.

<sup>b</sup>Inhibitor concentrations used were as follows: DPF, 1 mM; T-o-TP, 1 mM; PMSF, 5 mM; eserine, 1 mM; CMBSA, 2 mM; and acetazolamide, 20 mM. Two replicates of triplicate gels of esterases were analyzed for substrate and inhibitor specificity.

were most active against esters of acetic acid but also showed some activity toward  $\beta$ -naphthyl laurate and thiophenylacetate.

Table I also shows results of characterization of esterases from rabbit erythrocytes on the basis of stability to heat and urea. The acetylcholinesterases (I) were stable in buffer at 55 C for 7 min and were still rela-

tively active after 18 min at this temperature; however, they were rapidly inactivated by 10 M urea at room temperature. Acetylsterases (II) and the slow (V) and fast (VII) migrating arylesterases revealed intermediate values for heat resistance and showed urea lability similar to the acetylcholinesterases (I). Most of the slow carboxylesterases (III a, c) were relatively stable to both heat and urea; however, the band in region 1.8 - 2.2 cm (III b) was extremely heat labile but remained active in the presence of urea. The intermediate (IV) and fast carboxylesterases (VIII) behaved similarly to the acetylsterases (II).

The on-gel enzymatic activities of the multiple forms of esterases from washings and membranes of rabbit erythrocytes were essentially constant over the pH range 5.7-7.4. At pH 8.0, however, all of the bands were approximately one-fourth to one-half as active as those observed at the other pH values.

#### CONCLUSIONS

These data from qualitative techniques of gel electrophoresis suggest that membranes of rabbit erythrocytes contain a complex array of esterases. Although some differences exist in the esterase patterns of washings and membranes, many of the enzymes exhibited similar mobilities and had corresponding substrate specificities, susceptibilities to inhibitors, pH optima, and sensitivities to heat and urea. The similarities of the banding patterns of esterases in the two fractions suggest that corresponding constituents were present in the cytoplasm and membranes of erythrocytes.

This investigation was done to standardize techniques (enzyme extraction, gel electrophoresis, detection, and identification) for examining cytoplasmic and membrane esterases of rabbit erythrocytes. Application of the techniques yielded results with good reproducibility—i.e., uniformity in intensity of gel staining, and repeatable spacial arrangement and identification of esterase bands between experiments. The electrophoretic patterns that were established may serve as standards to which those from physiologically altered test animals (e.g., due to nutritionally imbalanced diets) can be compared.

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