ment of the *erythro* peak obtained in the preparation described above gave a molar rotation in 5 N HCl of + 59.6°, on the basis of ninhydrin determination of the concentration, in good agreement with the reported values of + 59.5° (see ref. 3) and +61.6° (see ref. 6) for *erythro*-γ-hydroxy-L-glutamate. The product is homogeneous on paper chromatography and is uncontaminated with residual NH_4^+ or DPN^+, present in the second incubation mixture. Approximately equal amounts of the *erythro* and *threo* forms are recovered, indicating the formation of equal or nearly equal quantities of the two isomers of α-hydroxy-γ-ketoglutarate in the enzymatic condensation reaction, in confirmation of the earlier observation[5] of non-stereospecific enzymatic cleavage of the keto acid. The procedure above has been used repeatedly to obtain both unlabeled and ^{14}C-labeled *erythro*- and *threo*-γ-hydroxy-L-glutamate. The overall yield of both isomers from glyoxylate, as the limiting initial reactant, has been about 40–50%.

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Department of Pharmacology,
St. Louis University,
School of Medicine,
St. Louis, Mo. (U.S.A.)

ELIJAH ADAMS
ALFRED GOLDSTONE

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The sulfhydryl content of some lactate dehydrogenases*

Recently evidence has been obtained for the involvement of sulfhydryl groups in the mechanism of action of a number of lactate dehydrogenases (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27)[1]. Since lactate dehydrogenase now appears to consist of 4 subunits[2,3], it is possible that S–S bridges are involved in holding the subunits together. Therefore, in order to clarify further the role of the SH groups, we thought it of value to determine the cysteine and cystine content of a number of lactate

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dehydrogenases. PFLEIDERER *et al.*[4] have demonstrated by *p*-chloromercuribenzoate titration and amino acid composition measurements that there are 14 moles of cysteine per 126 000 g of protein in lactate dehydrogenase of pig heart, and rat and rabbit muscle.

Beef-heart, chick-heart and chick-skeletal-muscle lactate dehydrogenase were prepared as described previously[1]. Beef and halibut skeletal-muscle lactate dehydrogenase were prepared according to PESCE *et al.*[5]. Rabbit-skeletal-muscle lactate dehydrogenase was obtained from C. F. Boehringer und Soehne, Mannheim (Germany) and was further purified by chromatography on a DEAE-cellulose column. All the enzymes used in this study showed only one band on electrophoresis. They represent pure-type enzymes with identical subunits. For example, the chicken heart enzyme represents the CH_4 described by CAHN *et al.*[2]. The molecular weight adopted was 135 000 for all the enzymes. The extinction coefficients used at 280 mμ were $1.8 \cdot 10^5$ for chick-heart lactate dehydrogenase, $2.2 \cdot 10^5$ for chick-skeletal-muscle lactate dehydrogenase, and $2.0 \cdot 10^5$ for all the other enzymes[5]. Urea and guanidine·HCl were purchased from Fisher Scientific Company. The urea and guanidine·HCl were recrystallized once or twice from methanol. All the other compounds used in this study were reagent grade. Glass-distilled water was used throughout. For the spectrophotometric assay of the cysteine content of the enzymes with *p*-chloromercuribenzoate, the increase in absorbancy at 250 mμ due to mercaptide formation was measured in a Zeiss spectrophotometer model PMQ II (ref. 6). 0.1–0.2 ml of enzyme ($(5–7 \cdot 10^{-3}$ μmoles) were added to a 3-ml cuvette containing 0.1 M sodium phosphate buffer (pH 6.9), $1 \cdot 10^{-4}$ M or $1.6 \cdot 10^{-4}$ M *p*-chloromercuribenzoate and 7.0 or 8.8 M urea. SWENSON AND BOYER[7] have demonstrated that the extinction coefficient of the mercaptide formed between *p*-chloromercuribenzoate.and SH compounds is different in concentrated urea solutions and in water. In this work, this difference has been determined with reduced glutathione and the correction factor so obtained was applied to the reaction between *p*-chloromercuribenzoate and the enzymes. For the amperometric titration of the SH groups of the enzymes with $HgCl_2$, an apparatus similar to that described by KOLTHOFF AND HARRIS[8] was used, the main difference being that the platinum electrode was vibrating instead of rotating. The microammeter used was obtained from Rubicon Company, Philadelphia, Pa. The sensitivity was 0.01 μA/mm of deflection. The final concentrations of reagents in the mixture used for amperometric titration were: 4.0 M guanidine·HCl, 0.15 M NH_4NO_3, 0.066 M Na_2SO_3 (when present), 0.01–0.02 μmole enzyme. All the reagents were dissolved in 0.1 M Tris–HCl buffer (pH 7.0). The final volume was 3.0 ml. Before addition of the enzyme and of Na_2SO_3, the mixture was outgassed with N_2 for 10–15 min. The titration was carried out by addition, under N_2 atmosphere, of 10-μl aliquots of 2.5 mM $HgCl_2$; the equivalence point was reached in 4–8 additions; after that, 3–5 more aliquots were added. As a control, a titration of serum albumin was carried out. 0.6–0.7 SH groups were titrated in the absence of Na_2SO_3, and 15–16 in its presence. This value is in good agreement with the values reported in the literature[9]. Performic acid oxidation was carried out at room temperature either by the method of HIRS[10] or by that of SCHRAM, MOORE AND BIGWOOD[11]. The resulting cysteic acid was measured by means of a Beckmann amino acid analyzer according to the procedure of MOORE, SPACKMAN AND STEIN[12].

Table I shows the experimental results. Values are given in moles of SH groups

per 135 000 g of protein as determined by spectrophotometric titration, amperometric titration with and without Na_2SO_3, and cysteic acid analysis. The last column gives the average figures, approximated to the nearest integer. The values obtained by amperometric titration have been multipled by 2 on the assumption that, under the experimental conditions employed, mercury acts as a divalent cation and therefore 1 atom of this element binds to 2 SH groups (ref. 13). The agreement with the values obtained by *p*-chloromercuribenzoate titration and by cysteic acid analysis confirms this assumption.

TABLE I

SH GROUP CONTENT OF LACTATE DEHYDROGENASES PER 135 000 g OF PROTEIN

The number of determinations is given in parentheses.

Enzyme	*p-Chloromercuribenzoate titration*	*$HgCl_2$ titration*		*Cysteic acid analysis*	*Average*
		with Na_2SO_3	*without Na_2SO_3*		
Chick heart	26.3 (4)	26.2 (5)	26.5 (3)	27.5 (3)	27
Beef heart	16.6 (4)	16.0 (1)	16.6 (1)	16.8 (2)	17
Halibut skeletal muscle	13.1 (2)	14.7 (3)	15.0 (4)		14
Chick skeletal muscle	26.4 (3)	25.3 (1)	25.9 (1)	26.1 (1)	26
Beef skeletal muscle	25.0 (3)	26.2 (2)	24.8 (2)	26.3 (2)	26
Rabbit skeletal muscle	16.7 (3)			15.7*	16

* Value taken from PFLEIDERER *et al.*[4] and recalculated for a molecular weight of 135 000.

Similar values of SH content have been found by amperometric titration with and without Na_2SO_3 for all the enzymes tested. This finding, together with the close agreement among the figures obtained by cysteic acid analysis and by the other two methods, rules out the existence of S–S groups in these proteins. PFLEIDERER *et al.*[4]. reached this same conclusion for the lactate dehydrogenases from rabbit muscle, pig heart, and rat muscle. It seems, therefore, that the subunits in lactate dehydrogenases are not held together by S–S bridges. This is consistent with the finding by APPELLA AND MARKERT[3] that beef-heart lactate dehydrogenase can be split into subunits by the action of 12.0 M urea or 5.0 M guanidine.

Our values of 16.7 moles of SH for rabbit-skeletal-muscle lactate dehydrogenase and 16 for the beef-heart enzyme for 135 000 g of protein are in close agreement with the values of 14 found by PFLEIDERER *et al.*[4] for 126 000 g of protein and of 8 cysteic acid residues found by MILLAR[14] for 72 000 g of protein. It is of interest that the cysteine content of the various vertebrate lactate dehydrogenases varies. Furthermore, the two chicken types have essentially the same number of cysteine moieties, whereas the content for the two beef enzymes is different. Although the chicken enzymes show no difference in cysteine content the amino acid compositions of the two types are considerably different[15]. These variations in cysteine content among the lactate dehydrogenases further indicate that evolutionary change has taken place in the lactate dehydrogenase molecule.

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Graduate Department of Biochemistry,
Brandeis University,
Waltham, Mass. (U.S.A.)

GIOVANNI DI SABATO
AMADEO PESCE
NATHAN O. KAPLAN

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TPN-specific isocitrate dehydrogenase in bovine cornea epithelium

During the course of our investigations on enzyme patterns of the main metabolic pathways in connective tissues[1] enzyme activities were assayed separately in epithelia and connective tissue of bovine cornea. We found an extremely high activity for TPN-specific isocitrate dehydrogenase (EC 1.1.1.42) in cornea epithelium.

Cow's eyes were sectioned out immediately after slaughtering. Samples of both tunica propria and epithelium were homogenized in 50 mM triethanolamine–HCl buffer (pH 7.5), 5 mM EDTA, using a Waring blendor. For determination of enzyme activity the 100 000 × *g* supernatant was used in a optical test under standard conditions[1]. The stepwise extraction along with increasing disintegration of cornea epithelium was carried out as described earlier[2].

The enzyme pattern of cornea epithelium shows an extremely high isocitrate dehydrogenase activity. It exceeds all other enzyme activities assayed in the same tissue (Fig. 1). The maximum isocitrate dehydrogenase activity can be assumed to be twice the measured activity, since Mg^{2+} was used instead of Mn^{2+} for activity determination. In all the tissues of both man and animal that have been investigated so far no comparable isocitrate dehydrogenase activity has ever been encountered (Table I).