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Haloacetol Phosphates. Identification of the Sulfhydryl Group of Rabbit Muscle Aldolase Alkylated by Chloroacetol Phosphate†

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ABSTRACT: Previous work has shown that chloroacetol phosphate, a reactive analog of dihydroxyacetone phosphate, competitively inhibits aldolase at pH 7.0 and irreversibly inactivates the enzyme at pH 10.0 *via* alkylation of one SH group per catalytic subunit. Present studies of the interactions of chloroacetol phosphate inactivated aldolase with glyceraldehyde 3-phosphate and dihydroxyacetone phosphate suggest that the reagent moiety occupies the binding site for dihydroxyacetone phosphate. To determine whether the SH group that is alkylated by chloroacetol phosphate is one of the same groups shown previously to be necessary for maximal aldolase activity, we have isolated the peptide containing the acetol phosphate moiety. The inactivated aldolase was treated with NaB^3H_4, thereby reducing the carbonyl of the incorporated reagent and introducing a stable, radioactive marker; the enzyme was then digested with trypsin. Isolation of the S-alkylated peptide was achieved by successive chromatography of the tryptic digest on Bio-Rad AG 50W-X2 and DEAE-cellulose. Its amino acid composition is identical with that of the peptide isolated previously that contains the SH group protected by substrate from a variety of modifications. Thus, results with a reactive substrate analog provide additional evidence for the presence of a SH group in the active-site region of rabbit muscle aldolase.

Chloroacetol phosphate inactivates both yeast and rabbit muscle aldolases *via* preferential alkylation of one sulfhydryl group per catalytic subunit (Lin *et al.*, 1971; Paterson *et al.*, 1972). The specificities of these modifications, the ability of substrate to protect against inactivation, and the demonstration with the muscle enzyme that the reagent is also a competitive inhibitor indicate that these enzymes contain a sulfhydryl group in the active-site region. Some role of sulfhydryl groups in the catalytic function of aldolase was first suggested by the studies of Rowley *et al.* (1964) concerning inactivation of the enzyme with chlorodinitrobenzene; the postulated nature of this role has varied from intimate involvement in the catalytic process (Horecker, 1970), to an auxiliary function (Steinman and Richards, 1970), to no function at all (Anderson, 1972). In order to understand the significance of the inactivation of aldolase by chloroacetol phosphate with respect to the possible role of sulfhydryls, particularly within the context of previously reported sulfhydryl modifications, we have identified the group that is most reactive toward chloroacetol phosphate.

Materials and Methods

Rabbit muscle aldolase with a specific activity of 7.5 units/mg was a product of Boehringer Mannheim Corp. Triose phosphate isomerase, glycerol phosphate dehydrogenase, dihydroxyacetone phosphate, DL-glyceraldehyde 3-phosphate, Fru-1,6-P_2, NADH, and glycylglycine were purchased from the Sigma Chemical Co. Other materials and vendors were as follows: trypsin treated with TPCK [L-(tosylamido-2-phenyl)ethyl chloromethyl ketone], Worthington Biochemical Corp., and NaB^3H_4, New England Nuclear Corp. Chloroacetol phosphate was synthesized according to the published procedure (Hartman, 1970).

Methods for quantitating protein concentrations, aldolase activity, and radioactivity were identical with those used previously (Paterson *et al.*, 1972). Peptide mapping was performed with slight modification (Paterson *et al.*, 1972) of the method described by Katz *et al.* (1959).

Amino Acid Analyses. Samples were hydrolyzed with 6 N HCl at 110° for 21 hr in sealed, evacuated (<50 μm) tubes. The hydrolysates were concentrated to dryness on a rotary

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TABLE I: Reaction of Chloroacetol Phosphate Modified Aldolase with Glyceraldehyde 3-Phosphate.[a]

Sample[b]	Enzyme Act. (Units/mg)	Phosphate (μmol/μmol of Subunit)[c]	Glyceraldehyde 3-Phosphate (μmol/μmol of Subunit)[c]
Aldolase	6.5	0.4	
Aldolase + glyceraldehyde 3-phosphate	1.3	1.4	1.0
Modified aldolase	1.2	1.6	
Modified aldolase + glyceraldehyde 3-phosphate	0.018	2.4	0.8

[a] Aldolase (4 mg/ml) in 0.024 M triethanolamine hydrochloride (pH 7.9) was incubated with 1 mM DL-glyceraldehyde 3-phosphate for 2 hr. The solutions were assayed for activity and dialyzed against 0.01 M sodium acetate (pH 7.0). Aliquots of the dialyzed solutions containing 0.5–1.0 mg of protein were digested with 2 mequiv of sulfuric acid at 180° for 2 hr. The digests were diluted with 1.0 ml of water, heated in a boiling-water bath for 20 min, and then assayed for inorganic phosphate by the method of Marsh (1959). [b] The preparation of these samples is described in the Materials and Methods section. [c] One subunit constitutes 40 mg of protein.

TABLE II: Reaction of Chloroacetol Phosphate Modified Aldolase with Dihydroxyacetone Phosphate.[a]

Sample[b]	Enzyme Act. (Units/mg)		Incorporation (cpm/mg)	
	Before Borohydride	After Borohydride	Total	Representative of Dihydroxyacetone Phosphate
Aldolase	6.5	5.9	4,500	
Aldolase + dihydroxyacetone phosphate	6.5	0.18	43,000	38,500
Modified aldolase	1.2	1.1	6,500	
Modified aldolase + dihydroxyacetone phosphate	1.2	0.12	15,500	9,000

[a] Aldolase (2 mg/ml) in 2 ml of 0.15 M sodium succinate (pH 6.0) at 4° with and without 3 mM dihydroxyacetone phosphate was treated with two 0.02-ml additions of 1 M NaB^3H_4 (0.02 mCi/μmol). The pH was maintained at 6.0 with 2 M acetic acid. After the protein solutions were assayed for aldolase activity, they were dialyzed against 0.01 M sodium acetate (pH 7.0) and assayed for protein and radioactivity. [b] The preparation of these samples is described in the Materials and Methods section.

evaporator and analyzed on a Beckman 120C analyzer according to Spackman *et al.* (1958).

Preparation of Chloroacetol Phosphate Inactivated, Trypsin-Digested Aldolase. To a 20-ml solution of aldolase (8 mg/ml, 0.2 mM in subunit) in 0.05 M sodium glycinate–1 mM EDTA (pH 10.0) was added 0.5 ml of 0.04 M chloroacetol phosphate, the pH of which was adjusted to 8.0 with 1 N NaOH just prior to use. After the aldolase activity decreased to 5% of its initial value (about 1 hr), the excess chloroacetol phosphate was decomposed by the addition of β-mercaptoethanol (0.01 M). The solution was then cooled to 4°, and after the addition of one drop of octyl alcohol to decrease foaming, the carbonyl group of the protein-bound acetol phosphate moiety was reduced with 7.8 mg of NaB^3H_4 (0.2 mCi/μmol). After a 30-min incubation period, 20 ml of 7 M guanidine hydrochloride–0.1 M sodium phosphate–3 mM EDTA (pH 8.0) and 1.2 ml of 1 M sodium iodoacetate (pH 8.0) were added to the protein solution, which was then incubated in the dark at room temperature for 20 min. After the addition of β-mercaptoethanol (0.1 M), the solution was dialyzed exhaustively against 0.1 M ammonium bicarbonate (pH 8.0) and then incubated with 1.5 mg of trypsin at 40° for 3 hr. The sample was lyophilized to dryness and dissolved in 15 ml of 0.05 M formic acid. The specific radioactivity of the modified aldolase, determined after tryptic digestion, was 172×10^6 cpm/μmol (160 mg). Native aldolase that was treated with NaB^3H_4 under the same conditions contained 5.4×10^6 cpm/μmol. The extent of incorporation of chloroacetol phosphate was 7.5 mol/mol of aldolase as measured by organic phosphate analyses and 6.8 mol/mol of aldolase as measured by the difference in carboxymethylcysteine content (determined with a Beckman Model 120C amino acid analyzer) in acid hydrolysates of native and modified enzyme. These values are consistent with those obtained previously by direct assay of free sulfhydryl content or by using ^{32}P-labeled reagent (Paterson *et al.*, 1972).

Preparation of Chloroacetol Phosphate Inactivated Aldolase for Studies Involving Its Interaction with Dihydroxyacetone Phosphate and Glyceraldehyde 3-Phosphate. Aldolase (16 mg/ml) was incubated with 2 mM chloroacetol phosphate until 20% of the initial enzymic activity remained (30 min) and reduced with unlabeled $NaBH_4$ as described in the preceding paragraph. A sample of native aldolase that had been incubated at pH 10.0 for 30 min was also treated with $NaBH_4$. The two samples were then dialyzed against 0.01 M sodium acetate (pH 7.0).

Results

Interaction of Chloroacetol Phosphate Modified Aldolase with Glyceraldehyde 3-Phosphate and Dihydroxyacetone Phosphate. Samples of native and modified aldolase were incubated with DL-glyceraldehyde 3-phosphate under conditions described by Lai *et al.* (1968). After 2 hr, about 20% of the initial enzymic activity remained. The extents of organic phosphate incorporation (Table I) show that modification of aldolase by chloroacetol phosphate does not impair its reaction with glyceraldehyde 3-phosphate.

The aldolase samples were also subjected to borohydride reduction in the presence of dihydroxyacetone phosphate (Horecker *et al.*, 1963). The conditions were those described by Rose and O'Connell (1969), except that NaB^3H_4 was used so that incorporation of dihydroxyacetone phosphate could be monitored easily. The data presented in Table II indicate that chloroacetol phosphate modified aldolase cannot form a Schiff base with dihydroxyacetone phosphate.

TABLE III: Amino Acid Composition of Labeled Peptide.[a]

Amino Acid	μmol Found	Molar Equiv	N11 Peptide[b]
Lys	0.032	1.1	1
His	None		
Arg	None		
CM-Cys	None		1
Asp	0.044	1.5[c]	1 (Asn)
Thr	0.004	0.14	
Ser	0.030[d]	1.03	1
Glu	0.029	1.0[e]	1 (Gln)
Pro	None		
Gly	0.031	1.07	1
Ala	0.087	3.0	3
Val	Trace		
Met	None		
Ile	None		
Leu	0.059	2.0	2
Try	Trace		
Phe	None		

[a] Of the radioactivity applied to the long column, 53% emerged coincident with aspartic acid, and 41% emerged coincident with a ninhydrin-positive peak (0.010 μmol assuming a color yield equivalent to leucine's) appearing between glutamic acid and glycine. These elution positions agree with those reported for the analogous cysteine derivatives obtained from glutathione (Lin *et al.*, 1971). [b] Sajgó (1969). [c] The sum of the excess aspartic acid and the component appearing after glutamic acid (see footnote *a*) account for 0.8 molar equiv of the alkylated cysteine. [d] Corrected assuming a 10% destruction during hydrolysis. [e] Arbitrarily set at 1.0 molar equiv.

Purification and Characterization of Chloroacetol Phosphate Labeled Peptide. A sample (25 mg in 3.5 ml of formic acid; 25.7×10^6 cpm) of the tryptic digest of aldolase inactivated with chloroacetol phosphate (see Materials and Methods section) was placed on a 1.5 × 25 cm column of Bio-Rad AG 50W-X2 ion-exchange resin at 50° equilibrated with 0.2 M pyridine–4.6 M acetic acid (pH 3.1). All buffers used in the purification contained 0.25% (v/v) thiodiglycol. The column was eluted with a linear gradient of 200 ml each of the equilibration buffer and 2.0 M pyridine–2.4 M acetic acid (pH 5.0). Two major radioactive peaks (I and II, Figure 1a) at a ratio of 2.2:1 were observed, which comprised 46 and 21% of the radioactivity applied to the column. The total recovery of radioactivity (summation of all collected fractions) was 88%. Fractions 10–13 were combined, lyophilized to dryness, and dissolved in 0.01 M ammonium acetate (pH 7.5). A portion (6×10^6 cpm) of this sample was applied to a 1.5 × 25 cm column of Whatman DEAE-cellulose (DE-52) equilibrated with 0.01 M ammonium acetate (pH 7.5). Elution of the column with a linear gradient of 250 ml each of the equilibration buffer and 0.25 M ammonium acetate (pH 7.5) gave one major radioactive peak containing 80% of the counts applied (Figure 1b). This peak (fractions 15–17), which was lyophilized to dryness, contained a single peptide as demonstrated by amino acid analysis (Table III) and peptide mapping (Figure 2). The position of the peptide on the map is the same as that of the major radioactive peptide detected previously in a digest of aldolase inactivated with chloroacetol [^{32}P]phosphate (Paterson *et al.*, 1972).

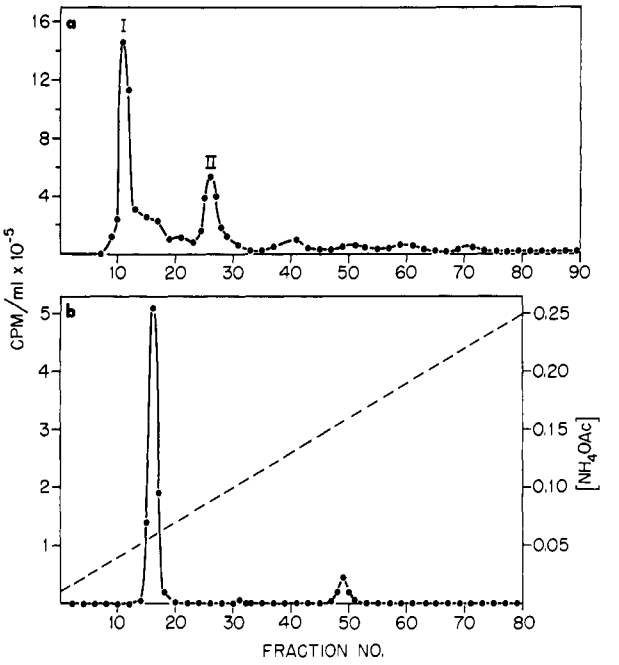

FIGURE 1: Purification of labeled peptide from a tryptic digest of aldolase inactivated with chloroacetol phosphate and subsequently reduced with NaB^3H_4. (a) Ion-exchange chromatography on Bio-Rad AG 50W-X2. The flow rate was 60 ml/hr; 3-ml fractions were collected and 0.01-ml aliquots were assayed for radioactivity. (b) Ion-exchange chromatography of peak I from part a on DEAE-cellulose. The flow rate was 20 ml/hr; 6-ml fractions were collected and 0.01-ml aliquots were assayed for radioactivity. Other details are described in the Results section.

A portion of peak II (Figure 1a) was chromatographed on DEAE-cellulose under conditions identical with those already described above. Multiple radioactive components were observed; therefore, no further attempts were made to characterize this material.

Discussion

In our previous report (Paterson *et al.*, 1972) concerning the reaction of chloroacetol phosphate with rabbit muscle aldolase, we were uncertain whether the reagent reacts with the binding site of glyceraldehyde 3-phosphate or of dihydroxyacetone phosphate. By the criteria of fluorescent quenching and protection against tryptic digestion (Rose and O'Connell, 1969), chloroacetol phosphate behaved as a dihydroxyacetone phosphate analog, but Schiff-base formation between reagent and enzyme could not be demonstrated with borohydride reduction. We have explored this question further by examining the reaction of chloroacetol phosphate inactivated aldolase with glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. If the acetol phosphate moiety resides at the dihydroxyacetone phosphate site, Schiff-base formation should be prevented, whereas if the glyceraldehyde 3-phosphate site is blocked, the covalent incorporation of this aldehyde *via* condensation with a protein sulfhydryl group (Lai *et al.*, 1968) should not occur. It is clear from the data (Tables I and II) that the aldolase modified with chloroacetol phosphate reacts with glyceraldehyde 3-phosphate to about the same extent as the native enzyme. In contrast, the modified enzyme is apparently incapable of forming a Schiff base with dihydroxyacetone phosphate. Some incorporation of the ketone substrate is observed, but only to an extent that is

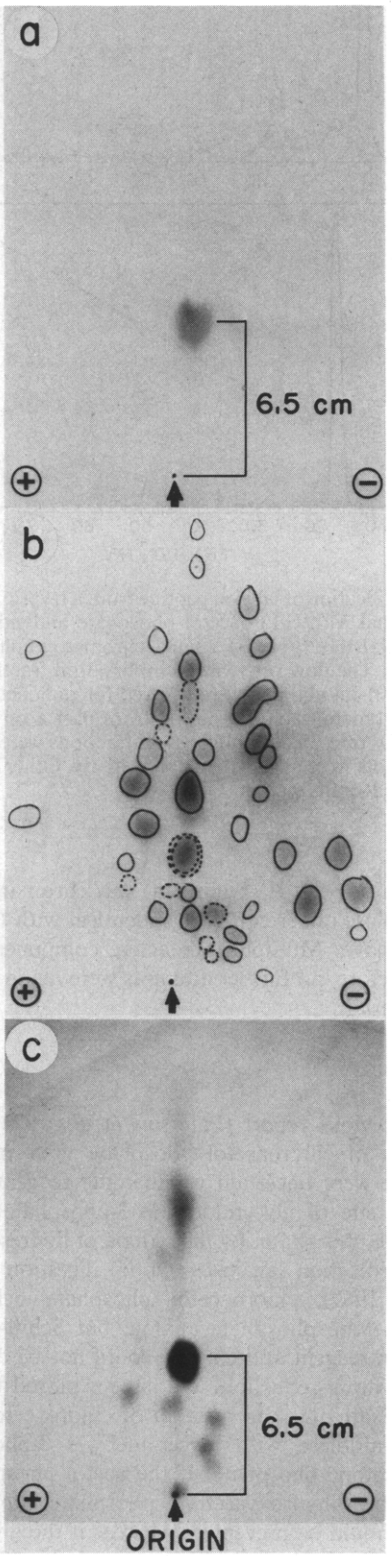

FIGURE 2: Peptide map (a) of purified peptide containing the chloroacetol phosphate alkylated sulfhydryl group. The radioactivity was found to be associated with the ninhydrin-positive spot by cutting it out and counting it in a liquid scintillation spectrometer. For comparative purposes, a peptide map (b) and autoradiogram (c) of a tryptic digest of aldolase after inactivation with chloroacetol [^{32}P]-phosphate and reduction with borohydride are included. Note that the purified peptide and the major ^{32}P-containing peptide coincide.

consistent with the enzymic activity of the modified aldolase as compared to that of the unmodified aldolase. The aldolase used in these experiments was only 80% inactivated so as to minimize random alkylation of sulfhydryl groups, which becomes more significant upon prolonged incubation with the reagent. We conclude that the sulfhydryl group alkylated by chloroacetol phosphate is close to the dihydroxyacetone phosphate binding site. Furthermore, the ability of the inactive enzyme to react with glyceraldehyde 3-phosphate argues against major conformational changes as a result of the alkylation.

The preferential alkylation of one sulfhydryl group per aldolase subunit by chloroacetol phosphates provides strong evidence for the presence of a sulfhydryl group in the vicinity of the dihydroxyacetone phosphate binding site, since (1) the alkylating agent is a dihydroxyacetone phosphate analog with a demonstrated affinity for the active site, and (2) the chloroacetol phosphate modified aldolase apparently has lost the capacity to bind dihydroxyacetone phosphate. Therefore, we felt that the identification of the alkylated sulfhydryl group would be of interest, particularly in view of the controversy concerning the role of sulfhydryl groups in aldolase activity.

The peptide containing the sulfhydryl group most reactive toward chloroacetol phosphate was purified to homogeneity by ion-exchange chromatography. Thirty-seven per cent of the radioactivity associated with the total tryptic digest was present in the purified peptide. This represents a good recovery, since it was previously estimated (Paterson *et al.*, 1972) that about 50% of the incorporated reagent was accounted for by the major site of alkylation. That the isolated peptide is identical to the one that was visualized by autoradiography when the inactivation was carried out with chloroacetol [^{32}P]phosphate was verified by peptide mapping (Figure 2). We also tested the assumption that the radioactivity of the isolated peptide was indicative of the presence of the reagent moiety, since rather than using labeled chloroacetol phosphate, the isotope was introduced *via* NaB^3H_4 subsequent to the inactivation. Most of the radioactivity in hydrolysates of the peptide emerged from the long column of the amino acid analyzer coincident with the cysteine derivatives obtained from chloroacetol phosphate alkylated glutathione (Lin *et al.*, 1971).

Amino acid analyses of the isolated peptide identify it as the one designated TR4 by Anderson and Perham (1970) and N11 by Sajgó (1969), who has determined its sequence. The apparently high value for aspartic acid is due to the coincident elution position of one hydrolysis product of the alkylated cysteine. The cysteinyl residue in the peptide corresponds to the residue in aldolase that is protected by substrate from modification by bromoacetate (Szajáni *et al.*, 1970), iodoacetamide (Anderson and Perham, 1970), dialkyl disulfide monosulfoxides (Steinman and Richards, 1970), and air oxidation (Lai *et al.*, 1971). With the exception of air oxidation, in which two sulfhydryls are oxidized simultaneously to form a disulfide bridge, this substrate-protected sulfhydryl group is not the most reactive. For example, in the case of inactivation of aldolase by bromoacetate, the most reactive sulfhydryl group is situated, in the primary sequence, close to the essential ϵ-amino group. Carboxymethylation of this group does not alter the enzymic activity; loss of activity parallels the subsequent alkylation of the sulfhydryl group found in peptide N11. Thus, the preferential alkylation by chloroacetol phosphate of a sulfhydryl group that is not inherently the most reactive toward general alkylating agents is further evidence that the reagent is acting as an active-site-directed compound.

If the sulfhydryl group labeled by chloroacetol phosphate is present at the dihydroxyacetone phosphate binding site, is it functional in catalysis? It clearly is not essential to catalysis, because Steinman and Richards (1970) have shown that an aldolase derivative in which this sulfhydryl group, in addition to three others, is present as a mixed disulfide is 50% as active as native aldolase. Also, Anderson (1972) has reported that aldolase from sturgeon muscle does not contain a sulfhydryl group corresponding to the one in the N11 peptide of the rabbit enzyme; *i.e.*, none of the cysteine-containing peptides isolated from a tryptic digest of the sturgeon enzyme are homologous with peptide N11 from rabbit aldolase. Therefore, the group alkylated by chloroacetol phosphate may merely be close to the active site, with no specific role in catalysis. The comparative studies by Anderson (1972), however, do not exclude an auxiliary role as suggested by Steinman and Richards (1970), for a residue with such a role would not necessarily be species invariant.

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Demonstration of the Heterogeneity of Nucleoside Diphosphokinase in Rat Tissues†

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ABSTRACT: Nucleoside diphosphokinase (NDP-kinase) activity ranging from 30 to 68 μmolar units per g was measured in the following rat tissues: erythrocytes, brain, liver, heart, lung, kidney, spleen. More than 70% of the activity in the rat liver cell was found in the supernatant fraction with the remainder of the activity distributed in the nuclear, mitochondrial, and microbody fractions. Marked heterogeneity of this enzyme upon electrofocusing was found in various tissues of rats and subcellular fractions of rat liver. Each tissue or subcellular fraction had its own characteristic NDP-kinase electrofocusing profile. Although most of the electrofocusing peaks are present in all the tissues or subcellular fractions of rat liver cells, there are marked differences in the relative quantities of the various electrofocusing peaks. The NDP-kinase electrofocusing profile of rat erythrocytes differs greatly from that reported earlier for human erythrocytes. Two isozymes isolated by electrofocusing a partially purified rat liver NDP-kinase were found to have distinct mobilities on agarose electrophoresis and different kinetic parameters with nucleotide substrates.

The occurrence of marked heterogeneity of NDP-kinase[1] in human erythrocytes has been demonstrated in previous studies (Cheng *et al.*, 1970, 1971). Six distinct peaks of enzymatic activity with isoelectric points ranging from 5.4 to 8.3 were observed in erythrocytes pooled from about 100 persons and from single individuals of different races. Studies with the individual electrophoretic peaks revealed molecular weights varying from 80,000 to about 100,000. Although the presumed isozymes can react with various di- or triphosphate nucleotides, marked differences in the kinetic parameters were seen. Arrhenius plots were linear for isozymes of pI 5.4, 5.8, 6.3, and 6.8 and were biphasic for isozymes of pI = 7.3 and 8.3. Marked differences in stability under various conditions were also observed (Cheng, 1972). Recently it was found that the heterogeneity of NDP-kinase is not unique for human erythrocytes but also occurs in erythrocytes of all species examined including those of elasmobranchs and cyclotomes (Cheng and Parks, 1972; Parks *et al.*, 1973). Although sev-

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[1] Abbreviation used is: NDP-kinase, nucleoside diphosphokinase.