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.

References

Anderson, P. J. (1972), *Can. J. Biochem. 50*, 111.

Anderson, P. J., and Perham, R. N. (1970), *Biochem. J. 117*, 291.

Hartman, F. C. (1970), *Biochemistry 9*, 1776.

Horecker, B. L. (1970), *in* Federation of European Biochemical Society Symposium, Vol. 19, Sols, A., and Grisolia, S., Ed., New York, N. Y., Academic, p 181.

Horecker, B. L., Rowley, P. T., Grazi, E., Cheng, T., and Tchola, O. (1963), *Biochem. Z. 338*, 36.

Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.

Lai, C. Y., Chen, C., Smith, J. D., and Horecker, B. L. (1971), *Biochem. Biophys. Res. Commun. 45*, 1497.

Lai, C. Y., Dretz, M., Bacila, M., Marinello, E., and Horecker, B. L. (1968), *Biochem. Biophys. Res. Commun. 30*, 665.

Lin, Y., Kobes, R. D., Norton, I. L., and Hartman, F. C. (1971), *Biochem. Biophys. Res. Commun. 45*, 34.

Marsh, B. B. (1959), *Biochim. Biophys. Acta 32*, 357.

Paterson, M. C., Norton, I. L., and Hartman, F. C. (1972), *Biochemistry 11*, 2070.

Rose, I. A., and O'Connell, E. L. (1969), *J. Biol. Chem. 244*, 126.

Rowley, P. T., Tchola, O., and Horecker, B. L. (1964), *Arch. Biochem. Biophys. 107*, 305.

Sajgó, M. (1969), *Acta Biochim. Biophys. Acad. Sci. Hung. 4*, 385.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Steinman, H. M., and Richards, F. M. (1970), *Biochemistry 9*, 4360.

Szajáni, B., Sajgó, M., Biszku, E., Friedrich, P., and Szabolcsi, G. (1970), *Eur. J. Biochem. 15*, 171.

Demonstration of the Heterogeneity of Nucleoside Diphosphokinase in Rat Tissues†

Yung-Chi Cheng, Bonnie Robison, and R. E. Parks, Jr.*

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-

† From the Section of Biochemical Pharmacology, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912. *Received July 10, 1972.* Supported by Grant CA 07340 from the National Institutes of Health, U. S. Public Health Service.

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

eral findings in other laboratories suggest that heterogeneity of NDP-kinase might exist in other tissues, there are no definitive reports of such isozymes. For example, Glaze and Wadkins, in a study of NDP-kinase from calf liver mitochondria, separated two peaks of activity by DEAE-cellulose chromatography (Glaze and Wadkins, 1967). In a study of NDP-kinase from calf thymus, Nakamura and Sugino separated two peaks of NDP-kinase activity by chromatography on DEAE-cellulose at pH 7.5. These fractions from thymus were indistinguishable with regard to specificity toward substrates, optimal pH, and requirement for divalent cations (Nakamura and Sugino, 1966). There have not been clear-cut demonstrations of the presence of NDP-kinase isozymes in other tissues.

These observations raise certain questions. Does heterogeneity of NDP-kinase occur in tissues other than the erythrocyte? If such heterogeneity occurs, what is the pattern of distribution of NDP-kinase isozymes among the subcellular fractions? Therefore, we have examined NDP-kinase isozyme patterns in various tissues of the rat and also in the subcellular fractions of rat liver cells.

Prior studies of NDP-kinases of human erythrocytes in this laboratory have shown that certain purification procedures, such as ion exchange chromatography, acetone fractionation, pH 5.0 precipitation, etc., can cause marked or total loss of certain isozymes (Cheng, 1972). Therefore, the present studies were performed with extracts either of crude homogenates or of cell fractions where the greatest possible recovery of total NDP-kinase activity occurs. In addition, isoelectric focusing experiments were performed with a partially purified NDP-kinase fraction from rat liver.

Materials and Methods

The disodium salt of glucose 6-phosphate was purchased from Boehringer-Mannheim. Cytochrome *c* and the disodium salt of phenyl phosphate were obtained from Sigma Chemical Co.; hydrogen peroxide was from B & A General Chemical Co. Sources of other reagents, the NDP-kinase assay procedure, definition of units of activity and the isoelectric focusing method have been described previously (Cheng *et al.*, 1971; Mourad and Parks, 1966a). Rats were purchased from Charles River Breeding Laboratory, Inc.

Preparation of Hemolysates of Rat Blood. The fresh blood from two individual rats was drawn in heparinized syringes. The erythrocytes were washed free of plasma and buffy coat by centrifuging in a clinical centrifuge three times in 0.9% sodium chloride solution. Erythrocytes were hemolyzed by addition of 4 volumes of distilled water, frozen, and thawed twice. The hemolysates were centrifuged to remove stroma. Prior to electrofocusing, hemolysates were dialyzed against 0.05 M Tris-acetate buffer (pH 7.5) for 2 hr.

Preparation of Crude Extracts of Rat Tissues. Heart, kidney, spleen, lung, brain, and isotonic saline-perfused liver of a rat were cut into small pieces and washed thoroughly with chilled physiological saline. Tissues were blotted with tissue paper. After weighing, a 10% (w/v) suspension was prepared for each tissue by homogenization in distilled water with a Potter-Elvehjem homogenizer, equipped with a smooth Teflon pestle. The homogenates were frozen and thawed three times. Membrane fragments were removed by centrifugation at 1000*g* for 10 min. The supernatant fluids obtained from the different tissues were measured for enzymatic activity and dialyzed against 1% pH 5–8 Ampholine solutions for 2 hr before being placed in the isoelectrofocusing column. Tissue homogenates were all prepared the same day and were stored frozen until the electrofocusing procedure. There was usually a 5–10% loss of the original NDP-kinase activity within 1 week.

Fractionation of Rat Liver Cells. The procedure for the fractionation of rat liver cells is that described by Van Lancker (Walkinshaw and Van Lancker, 1964). After separating the cells into five subcellular fractions, the enzymes in each fraction were solubilized by freezing and thawing three times. Enzymatic activity was measured before and after centrifugation at 10,000*g* for 10 min. After dialysis against 0.05 M, pH 7.5 Tris–acetate buffer, the samples were used for electrofocusing. The fact that adequate separation of the different subcellular fractions of the rat liver cells was achieved was demonstrated by measuring the activity or concentration of each biochemical marker in each subcellular fraction. The biochemical markers used were: nuclei, DNA; mitochondria, cytochrome oxidase; endoplasmic reticulum, glucose-6-phosphatase; microbodies and lysosomes, acid phosphatase; and supernatant fluid, catalase.

Partial Purification of Rat Liver NDP-kinase. A procedure was devised for partial purification of rat liver NDP-kinase that would give the greatest possible total recovery of activity prior to electrofocusing. Eight grams of rat liver, freed of blood by perfusion, was frozen at −20° overnight. After thawing it was homogenized with a Potter-Elvehjem homogenizer in five volumes of cold distilled water. After 30 min the suspension was centrifuged at 10,000*g* for 10 min, and the supernatant fluid was decanted (NDP-kinase activity, 520 units; specific activity 0.48 unit/mg of protein). To 20 ml of this supernatant fluid was added 20 ml of calcium phosphate gel prepared by the method of Tsuboi and Hudson (1957) (43 mg/ml dry wt). The suspension was stirred for 30 min and centrifuged at 10,000*g* for 15 min. No NDP-kinase activity was detected in the supernatant fraction which was discarded. The calcium phosphate precipitate was extracted with 7.0 ml of an ammonium sulfate solution at 20% of saturation adjusted to pH 7.5 with ammonium hydroxide. A total of 224 units of NDP-kinase activity was obtained (84% yield; specific activity 1.8 units/mg of protein). Thus, approximately fourfold purification was achieved with excellent recovery of total enzymic activity. The ammonium sulfate extract was dialyzed overnight against two changes of 100 ml each of 0.5% Ampholine solution (pH 5–8). No loss of activity occurred during dialysis. The dialyzed solution was employed directly in isoelectric focusing.

Results

NDP-kinase Activities of Different Tissues of the Rat. NDP-kinase activity in rat tissues was measured before and after the final centrifugation which was used to remove membrane fragments and denatured proteins. There was no detectable loss of NDP-kinase activity in any of the tissues examined indicating that the NDP-kinases in these tissues are not membrane-associated proteins. Table I shows the NDP-kinase activities in different tissues of rat after centrifugation. It can be seen that the enzymatic activities per gram of tissue of spleen, heart, liver, brain, and kidney are similar. The enzymatic activity of erythrocytes is relatively low but higher than that of lung. No significant difference in the levels of NDP-kinase activity was found in tissues from rat to rat. Measurements were repeated at least three times for each rat tissue. The specific activity of the enzyme from each tissue is also shown in Table I. It should be noted that the activities are all high and relatively constant from one tissue to the next.

TABLE I: Activities of Nucleoside Diphosphate Kinase in Different Tissues of Rat.

Tissue	Enzymatic Act. (μmolar Units/g of Tissue)	Sp Act. (μmolar Units/mg of Protein)
Erythrocyte	47	0.31
Spleen	63	0.16
Heart	68	0.87
Liver	62	0.43
Kidney	62	0.38
Lung	30	0.24
Brain	64	0.84

Electrofocusing Profiles of NDP-kinase from Various Tissues of the Rat. Figures 1 and 2 present typical electrofocusing profiles of NDP-kinases from various tissues of the rat. Each experiment was repeated at least twice. The heterogeneity of NDP-kinase in electrofocusing occurs in all tissues surveyed and each tissue has its own characteristic NDP-kinase isozyme pattern. In all rat tissues examined, except erythrocytes, multiple distinct NDP-kinase enzymatic peaks were observed with isoelectric points of 5.3, 5.5, 5.65, 5.8, 6.05, and 6.55 (Figures 1 and 2). The peak at pH 6.55 is missing from the electrofocusing profile of erythrocytes of the rat (Figure 1A). It is of interest that the electrophoretic profile of rat erythrocytes differs markedly from that of human erythrocytes described previously (Cheng *et al.*, 1971). The isozymic peaks at pH's 8.3, 7.3, and 6.8, which are very prominent in human erythrocytes, are absent from rat erythrocytes. With liver, heart, and erythrocytes, no difference in the NDP-kinase electrofocusing profile between dialyzed and the non-dialyzed samples was observed. The relative amounts of isozyme under each peak vary from one tissue to another, *e.g.*, about 51% of the heart NDP-kinase activity appears under the peak at pH 6.55, whereas only 3% of the brain NDP-kinase activity occurs in this region.

Since tissues such as liver, heart, kidney, brain, and spleen have approximately the same amount of NDP-kinase activity per gram of tissue, a direct comparison of the enzymatic activities of each peak would be interesting. However, due to insufficient resolution of the heterogeneous NDP-kinase patterns upon electrofocusing over a wide pH range, it is not yet possible to measure the ratio of the enzymatic activity of each peak to the total activity of that tissue. It may be possible, however, that refocusing of discrete regions of the profiles will offer greater resolution of the individual patterns.

We have observed unusual stability of NDP-kinases during the 2–3 days required for electrofocusing and assay which suggests that the presence of Ampholine may stabilize the enzyme. It is likely, however, in view of the varied stability of the different isozymes, that somewhat different profiles may be seen if conditions other than those used in this study are employed.

Subcellular Localization of NDP-kinase Activities in Rat Liver. When the activity of NDP-kinase solubilized by freezing and thawing of the subcellular fractions of rat liver was measured, 74% of the total activity was found in the supernatant fraction, 12% in the nucleus, 7% in the mitochondrial fraction, 5% in the fraction of microbodies and cytosomes, and only negligible activity in the endoplasmic reticulum fraction (Table II). This experiment has been repeated with very similar results. Since enzymatic activity was found in all of the four subcellular fractions other than endoplasmic reticulum, it was of interest to learn whether the NDP-kinase isozymic patterns from various subcellular fractions differ from each other.

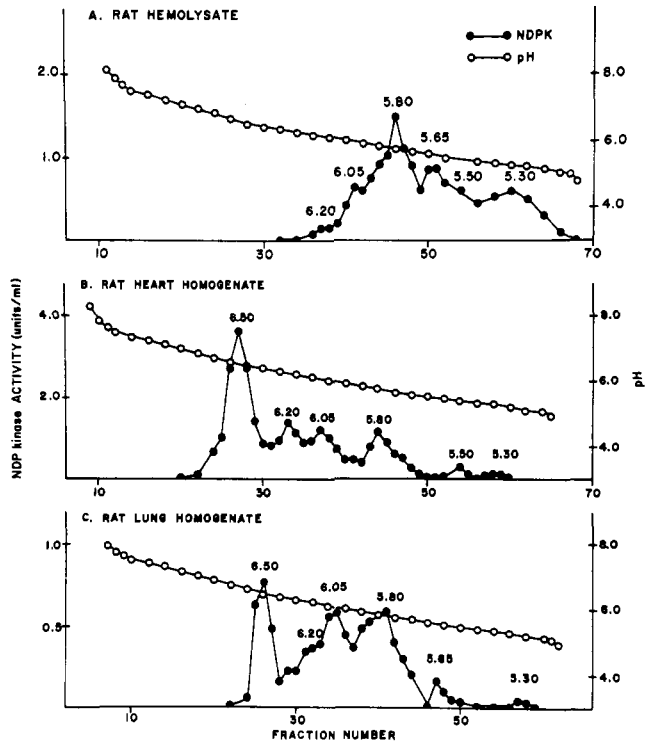

FIGURE 1: Electrofocusing profiles of NDP-kinase from rat hemolysates and from rat heart and lung homogenates. Electrofocusing was run for 60 hr at 600 V with pH 5–8 Ampholine in a 110-ml LKB electrofocusing column. The contents of the column were collected in fractions of 1.3 ml and were assayed for NDP-kinase enzymatic activity by the coupled pyruvate kinase–lactic dehydrogenase method. In part A, the dialyzed, centrifuged rat hemolysates containing 20 units of NDP-kinase activity were prepared as described in Materials and Methods. In part B, rat heart homogenate with approximately 15 units of NDP-kinase activity, and in part C, rat lung homogenate with approximately 15 units of NDP-kinase activity were processed and run for electrofocusing. Both samples, B and C, were stored in the freezer for 60 hr before being applied to the column.

TABLE II: Per Cent of NDP-kinase Activity in Various Subcellular Fractions of Rat Liver Cell.[a]

Subcellular Fraction	% Enzymatic Act.
Nuclei	12
Mitochondria	7
Cytosomes and microbodies	5
Endoplasmic reticulum	Nil
Supernatant	74

[a] Preparation of various subcellular fractions and assays for NDP-kinase activity are described in Materials and Methods. Enzymatic activity of each fraction was measured, corrected for dilution, multiplied by the dilution factor, and then divided by the total activity of all the fractions.

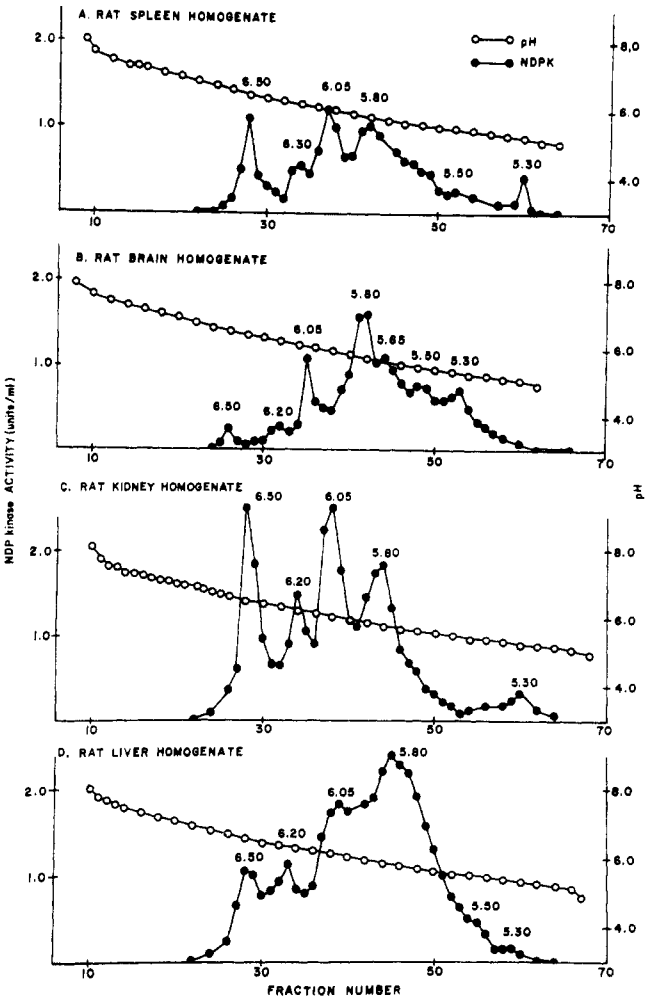

FIGURE 2: Electrofocusing profiles of NDP-kinase from rat spleen, brain, liver, and kidney homogenates. The dialyzed homogenate from each tissue was prepared as described in Materials and Methods. Conditions for electrofocusing are described in Figure 1. In part A, rat spleen homogenate with approximately 20 units of NDP-kinase activity, and in part B, rat brain homogenate with approximately 20 units of NDP-kinase activity were processed. Both samples were stored in the freezer for 5 days before being placed on the electrofocusing column. In part C, kidney homogenate with 40 units of enzymatic activity, and in part D, liver homogenate with approximately 40 units of enzymatic activity were used.

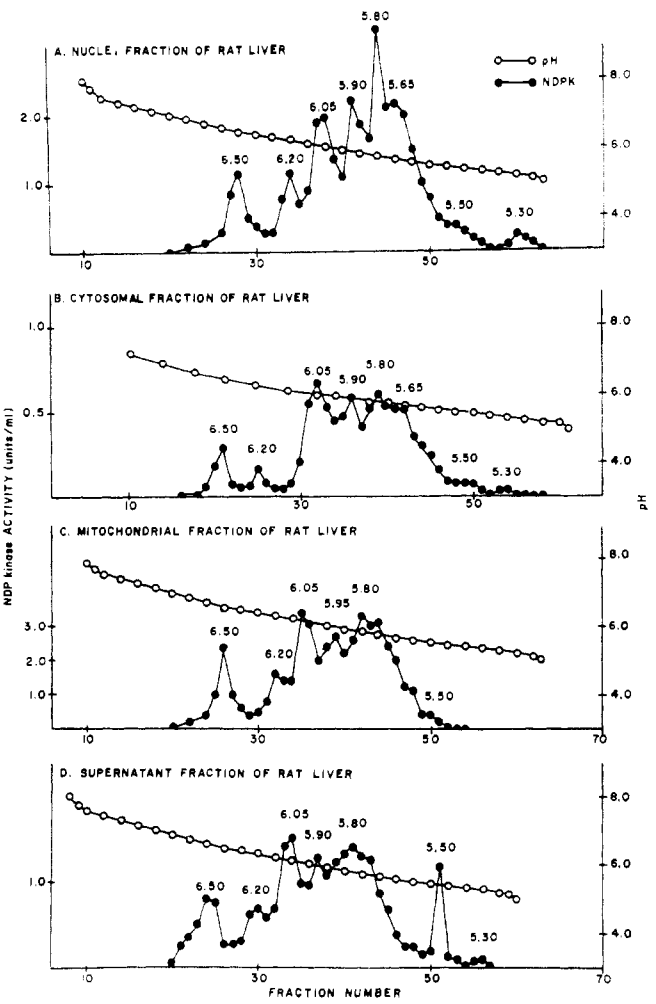

FIGURE 3: Electrofocusing profiles of NDP-kinase from nuclear, cytosomal, mitochondrial, and supernatant fractions of rat liver cell. The procedures for fractionating the rat liver cell and the assay for NDP-kinase activity are described in Materials and Methods. Electrofocusing procedures are described in the legend for Figure 1. In part A, dialyzed nuclear fractions of rat liver cell with about 40 units of NDP-kinase activity, and in part B, dialyzed cytosomal and microbody fractions with about 10 units of NDP-kinase activity were stored frozen for three days before electrofocusing. In part C, dialyzed mitochondrial fractions of rat liver with 50 units of NDP-kinase activity, and part D, dialyzed supernatant fractions of rat liver with about 25 units of enzymatic activity were applied to the electrofocusing column immediately after preparation of the samples.

Isoelectrofocusing Profiles of NDP-kinases from Various Subcellular Fractions of Rat Liver. Figure 3 presents the electrofocusing profiles of NDP-kinase activity from four subcellular fractions of the rat liver. Heterogeneity of the NDP-kinase activity occurs in all four fractions examined. The enzymic peak at pH 5.9 was not apparent in the electrofocusing profile of the whole homogenate of liver (Figure 2D) whereas it was clearly seen in the nuclear, mitochondrial, and cytosomal fractions (Figure 3A–C). It is noteworthy that the amount of enzymatic activity at pH 5.5 relative to the total enzymatic activity in the supernatant fraction (Figure 3D) is much higher than that in the nuclear, mitochondrial, or cytosomal and microbody fractions.

The total amount of NDP-kinase activity varies markedly in the different subcellular fractions (Table II), and the isozyme patterns appear distinct and separate for each fraction. However, since definite peaks occur at certain pH values, *e.g.*, pH 6.5, 6.2, 5.8, it is possible that these isozymes are similar although the relative amounts seem to differ from one fraction to the next. Of interest is the observation that although the supernatant fraction contains 74% of the total NDP-kinase activity of liver homogenate, the clear, sharp peak of activity at pH 5.5 in the supernatant fraction (Figure 3D) is not seen in the pattern of the whole homogenate of liver (Figure 2D). It should be noted that in the isoelectric focusing experiments with whole homogenate, some precipitation occurred in the region of pH 5–6. Therefore, the possibility exists that the pH 5.5 peak seen in the supernatant fraction is inactivated or becomes obscured by the large shoulder seen in experiments with whole homgenates of liver.

Electrofocusing of Partially Purified Liver NDP-kinase. When 2.5 ml of liver NDP-kinase, purified about 4-fold as described in "Materials and Methods," was subjected to isoelectric focusing over a pH range of 5–8, the pattern of Figure 4 was obtained. When this isozyme profile is compared

TABLE III: Some Kinetic Parameters of the Two Isolated Rat Liver NDP-kinase Isozymes.

Isozyme	K_m Value $\times 10^3$ M					V(ATP)[c]: V(GTP)
	ATP[a]	GTP[b]	UTP[a]	ADP[a]	dTDP[a]	
6.05	0.40	0.11	0.50	0.050	0.40	4.00
6.50	0.12	0.10	0.13	0.015	0.50	2.75

[a] The corrected K_m values were determined by a method similar to that described by Mourad and Parks (1966a). [b] The apparent K_m was determined with a fixed concentration of dTDP at 0.4 mM. [c] V_{max} values of the varying substrates ATP or GTP were determined at fixed concentrations of dTDP at 0.4 mM for each isozyme by a method similar to that described by Mourad and Parks (1966a).

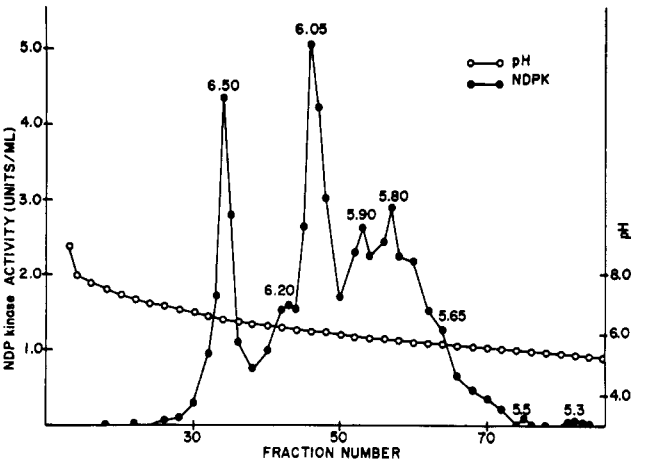

FIGURE 4: Electrofocusing profile of partially purified NDP-kinases from rat liver. The partially purified rat liver NDP-kinases were prepared as described in Materials and Methods. Approximately 70 units of NDP-kinase activity was used for electrofocusing. Conditions for electrofocusing were the same as described in Figure 1 except 1-ml fractions were collected.

with that for whole rat liver homogenate (Figure 2D), similar heterogeneity patterns are observed. However, with the partially purified NDP-kinase sample, substantially better resolution of the isozymes was obtained. It is of interest that similar peaks occur in both patterns indicating that the purification procedure used did not cause significant loss of any of the isozymes. Since excellent separation of activity was obtained with two of the isozymic peaks (pH 6.5 and 6.05), several additional experiments were performed with these isozymes.

When aliquots from the pI = 6.5 and 6.05 peaks were subjected to agarose electrophoresis with a specific overlay technique for detecting NDP-kinase activity, modified slightly from the method described previously (Cheng *et al.*, 1971), single bands of enzymic activity were observed that migrated with different mobilities. Table III presents the results of kinetic studies performed with the pI = 6.5 and 6.05 isozymes. Initial velocity experiments were performed with the coupled pyruvate kinase–lactate dehydrogenase and hexokinase–glucose-6-phosphate dehydrogenase assays (Mourad and Parks, 1966a). In these experiments, dTDP and ADP were the variable substrates and ATP and UTP were the changing fixed substrates. In each case, families of parallel lines were obtained which is consistent with the occurrence of a Ping-Pong reaction sequence (Cleland, 1963) similar to that described earlier for other NDP-kinases (Cheng *et al.*, 1971; Mourad and Parks, 1965; Garces and Cleland, 1969; Goffeau *et al.*, 1967).

It may be seen in Table III that the kinetic parameters with the two isozymes differ for individual nucleotide substrates by as much as threefold. In addition, there appear to be differences in the relative V_{max} values between ATP and GTP for the two isozymes.

Discussion

NDP-kinase activity is found to be high in all tissues of the rat examined. Membrane-free extracts of the homogenates in all cases had almost the same total NDP-kinase activity as the whole homogenate, suggesting that NDP-kinase from various tissues is a soluble enzyme rather than a membrane-bound protein. The levels of enzymic activities in various tissues reported in this paper are somewhat greater than those reported earlier (Mourad and Parks, 1966a). Several factors could be responsible for this observation. The use of a different substrate in the assay system might have led to differences in activity measurements. In the assay employed in the earlier study, the substrate dGDP was not used in concentrations much higher than its K_m value because of excessive reaction with the pyruvic kinase indicator system. The use of dTDP in more recent studies by this laboratory (Cheng *et al.*, 1971) overcame this problem. It should also be noted that the human erythrocytic NDP-kinase isozymes, although all are nonspecific for nucleotide substrates, differ markedly in their kinetic parameters with different nucleotides (Cheng *et al.*, 1971). Therefore it is possible that the assay employed in the present study did not measure the total maximal veloxity of all of the isozymes detected. Perhaps the total maximal NDP-kinase velocities in the different tissues and subcellular fraction are, in reality, somewhat higher than the values of this report. The differences in the two reports might also be due in part to differences in methods of preparation of the tissue extracts. The results described here are reproducible for preparation of various homogenates from different rats of the same strain, age, and sex.

The present findings demonstrate that the electrophoretic heterogeneity of the NDP-kinase is not confined to erythrocytes of various vertebrates (Cheng *et al.*, 1971; Cheng and Parks, 1972) but also occurs in a number of tissues of the rat and in the subcellular fractions of rat liver cells. There have been numerous reports of the enzymatic behavior of NDP-kinase at various levels of purity from a wide variety of tissues (Agarwal and Parks, 1971; Colomb *et al.*, 1966; Edlund, 1971; Glaze and Wadkins, 1967; Nakamura and Sugino, 1966; Miller and Wells, 1971; Ratliff *et al.*, 1964; Sedmak and Ramaley, 1971). Few, if any, of these earlier studies have considered the question of the marked heterogeneity of this enzyme. For example, a number of reports have appeared on NDP-kinases isolated from mitochondria of various tissues (Colomb *et al.*, 1966; Glaze and Wadkins, 1967; Goffeau *et al.*, 1967; Norman *et al.*, 1965; Pedersen, 1968). As seen in Figure 3C, we can readily identify at least five peaks of NDP-kinase activity by electrofocusing extracts of rat liver mitochondria. In view of the fact that the individual human erythrocytic NDP-kinase isozymes differ markedly in many parameters, *i.e.*, kinetic constants with nucleotide

substrates, temperature dependence, molecular weights, response to pH, etc. (Cheng *et al.*, 1971; Cheng, 1972), it seems likely that the NDP-kinase isozymes of other tissues will also be found to vary markedly in their enzymatic behavior and physicochemical properties. Therefore, in the future it will be necessary for investigators who wish to study NDP-kinases to establish which specific isozyme they are examining.

Since the electrofocusing profiles of the NDP-kinase extracts of various tissues are reporoducible in several rats of the same strain, age, and sex, these profiles appear to be characteristic of the tissue and subcellular fraction within a given species. However, a comparison of the NDP-kinase electrofocusing profiles of erythrocytes from a wide range of species has revealed marked variations which suggests that the NDP-kinases of a tissue may differ from one species to the next (Cheng and Parks, 1972; Parks *et al.*, 1973).

Although many of the electrofocusing peaks are present in all the various tissues and subcellular fractions of rat liver cells, there are striking differences in the relative amounts of each peak.

The high activity of NDP-kinase in all tissues examined indicates that if the enzyme, in fact, functions to exchange high-energy phosphate bonds rather indiscriminately among the various di- and triphosphate nucleotide species, a very rapid equilibration of the different nucleotides should occur and the ratio of ATP to ADP should be reflected in the tri- to diphosphate ratios of the non-adenine nucleotides. NDP-kinases, therefore, may be under some kind of allosteric or kinetic regulation. The occurrence of inhibitory abortive complexes with diphosphate nucleotides (Colomb *et al.*, 1966; Goffeau *et al.*, 1967; Mourad and Parks, 1966a) and responsiveness to the adenylate energy charge (Thompson and Atkinson, 1971) have been reported suggesting that the activities measured *in vitro* may not represent true activities in the cell. A recent study of the acid-soluble nucleotide patterns of erythrocytes of a number of species revealed marked differences between the ATP:ADP and GTP:GDP ratios which are inconsistent with the high activities of NDP-kinase found in these cells (Brown *et al.*, 1972). In all of the cases examined to date, NDP-kinase functions through the intermediation of a high-energy phosphorylated enzyme. It is possible that the activities assayed as NDP-kinases are, in fact, a family of enzymes which have other functions in metabolism. However, since these enzymes have low specificities for nucleotides, and all can react reversibly with triphosphate nucleotides to form enzyme–phosphate linkages (*e.g.*, 1-phosphohistidine) (Mourad and Parks, 1966b; Sedmak and Ramaley, 1971; Wålinder, 1968, 1969), they appear to be NDP-kinases. These considerations, along with the occurrence of the marked heterogeneity of the enzyme in nature, indicate that the true role of NDP-kinase is not established and is open to serious question. A detailed study of the properties of each electrofocusing enzyme peak may help clarify the physiological functions of NDP-kinases in the cell.

The fact that marked heterogeneity was observed with a partially purified preparation of NDP-kinase from rat liver and that the isozyme pattern (Figure 4) resembled that from the whole liver homogenate (Figure 2D) lends confidence to the use of these relatively crude fractions for the detection of NDP-kinase heterogeneity in various tissues and subcellular fractions. The observation that excellent recovery of total NDP-kinase activity was obtained by the use of the relatively mild calcium phosphate gel adsorption procedure, coupled with the great usefulness of this method in the purification of erythrocytic enzymes (Agarwal *et al.*, 1970; Cheng *et al.*, 1971), suggests that this method may have general application for the study of NDP-kinases in other tissues as well. Since the two purified liver isozymes yielded single bands of activity on agarose electrophoresis that have different mobilities, it appears that aggregation and nonspecific binding to other proteins is not a significant factor in the production of the obseved patterns of heterogeneity. The fact that these two isozymes displayed significantly different kinetic parameters with nucleotide substrates suggests that they do not represent simple variants of the same enzyme.

References

Agarwal, R. P., and Parks, R. E., Jr. (1971), *J. Biol. Chem. 246*, 2258.

Agarwal, R. P., Scholar, E. M., Agarwal, K. C., and Parks, R. E., Jr. (1970), *Biochem. Pharmacol. 20*, 1971.

Brown, P. R., Agarwal, R. P., Gell, J., and Parks, R. E., Jr. (1972), *Comp. Biochem. Physiol. 40B*, 777.

Cheng, Y.-C. (1972), Ph.D. Thesis, Brown University.

Cheng, Y.-C., Agarwal, R. P., and Parks, R. E., Jr. (1970), 2nd Northeast Regional Meeting of the American Chemical Society, Providence, R. I., p 63.

Cheng, Y.-C., Agarwal, R. P., and Parks, R. E., Jr. (1971), *Biochemistry 10*, 2139.

Cheng, Y.-C., and Parks, R. E., Jr. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 847.

Cleland W. W. (1963), *Biochim. Biophys. Acta 67*, 104, 173, 188.

Colomb, M. G., Laturaze, J. G., and Vignais, P. V. (1966), *Biochim. Biophys. Acta 67*, 188.

Edlund, B. (1971), *Acta Chem. Scand. 25*, 1370.

Garces, E., and Cleland, W. W. (1969), *Biochemistry 8*, 633.

Glaze, R. P., and Wadkins, C. L. (1967), *J. Biol. Chem. 242*, 2139.

Goffeau, A., Pedersen, P. L., and Lehninger, A. L. (1967), *J. Biol. Chem. 242*, 1845.

Miller, L. K., and Wells, R. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2298.

Mourad, N., and Parks, R. E., Jr. (1965), *Biochem. Biophys. Res. Commun. 19*, 312.

Mourad, N., and Parks, R. E., Jr. (1966a), *J. Biol. Chem. 241*, 271.

Mourad, N., and Parks, R. E., Jr. (1966b), *J. Biol. Chem. 241*, 3838.

Nakamura, H., and Sugino, Y. (1966), *J. Biol. Chem. 241*, 4917.

Norman, A. W., Wedding, R. T., and Black, M. K. (1965), *Biochem. Biophys. Res. Commun. 20*, 703.

Parks, R. E., Jr., Brown, P. R., Cheng, Y.-C., Agarwal, K. C., Kong, C. M., Agarwal, R. P., and Parks, C. C. (1973), *Comp. Biochem. Physiol.* (in press).

Pedersen, P. L. (1968), *J. Biol. Chem. 243*, 4305.

Ratliff, R. L., Weaver, R. H., Lardy, H. A., and Kuby, S. A. (1964), *J. Biol. Chem. 239*, 301.

Sedmak, J., and Ramaley, R. (1971), *J. Biol. Chem. 246*, 5365.

Thompson, F. M., and Atkinson, D. E. (1971), *Biochem. Biophys. Res. Commun. 45*, 1581.

Tsuboi, K. K., and Hudson, P. B. (1957), *J. Biol. Chem. 224*, 879.

Wålinder, O. (1968), *J. Biol. Chem. 243*, 3947.
Wålinder, O. (1969), *J. Biol. Chem. 244*, 1065.
Walkinshaw, C. H., and Van Lancker, J. L. (1964), *Lab. Invest. 13*, 513.

Ribulose Diphosphate Oxygenase. I. Synthesis of Phosphoglycolate by Fraction-1 Protein of Leaves†

T. J. Andrews,‡ G. H. Lorimer,§ and N. E. Tolbert*

ABSTRACT: Preparations of ribulose diphosphate carboxylase (fraction-1 protein) from both soybean and spinach leaves catalyzed the formation of phosphoglycolate and 3-phosphoglycerate from ribulose 1,5-diphosphate in the presence of oxygen. A manometric assay was used, and the activity called ribulose diphosphate oxygenase. Fraction-1 protein was purified from spinach leaves by a two-step procedure involving DEAE-cellulose chromatography and sucrose density gradient centrifugation in a zonal rotor. The protein was electrophoretically homogeneous. The oxygenase and carboxylase activities co-purified, and other attempts to separate them were unsuccessful. However, the oxygenase was more stable than the carboxylase, and the activity ratio, oxygenase/carboxylase, increased from 0.25 in the crude extract to 0.59 in the final product. The oxygenase was also more stable than the carboxylase when the protein was stored as an $(NH_4)_2SO_4$ precipitate. The pH optimum of the oxygenase activity was about 9.3–9.5, being much more alkaline than that of the carboxylase. No activity was observed in the absence of Mg^{2+} ions. A gas phase of 100% oxygen was not sufficient to saturate the oxygenase and the activity in air was 37% of that in pure oxygen. The Michaelis constant for ribulose 1,5-diphosphate was about 0.18 mM. The purified protein did not catalyze the oxygenation of several other phosphate esters. It is probable that, during photosynthesis, ribulose diphosphate is carboxylated and oxygenated by the same protein and that the oxygenating activity is responsible for the supply of phosphoglycolate, the first intermediate in the glycolate pathway of photorespiration.

It is now established that the glycolate pathway (Tolbert, 1963, 1971) is the metabolic sequence responsible for the physiological phenomenon of photorespiration in plants. This light-dependent uptake of oxygen and release of recently fixed carbon, as CO_2, accompany photosynthesis in atmospheres containing oxygen (Jackson and Volk, 1970). While the reactions involved in the metabolism of glycolate and the release of CO_2 are reasonably well understood, the mechanism of synthesis of this two-carbon acid has remained enigmatic ever since it was recognized as one of the major radioactive products of photosynthetic $[^{14}C]CO_2$ fixation (Benson and Calvin, 1950). Hypotheses concerning possible synthetic mechanisms may be grouped into three categories, namely (a) those which involve a reductive condensation of two molecules of CO_2 (reviewed by Zelitch, 1971), (b) those which propose the oxidation of the two-carbon fragment of dihydroxyethylthiamine pyrophosphate which is involved in the transketolase reaction (reviewed by Gibbs, 1969), and (c) the proposal of Ogren and Bowes (1971) that RuDP[1] is oxidized by molecular oxygen in the presence of RuDP carboxylase (fraction-1 protein), thus producing phosphoglycolate and 3-phosphoglycerate. Data in this paper and in our other recent publications (Andrews *et al.*, 1971; Lorimer *et al.*, 1972, 1973) substantiate the last hypothesis, which is consistent with a wide range of experimental observations concerning photorespiration and glycolate biosynthesis (see Discussion). Bowes *et al.* (1971) had obtained indirect evidence for phosphoglycolate formation by assaying for the glyoxylate phenylhydrazone, after incubation with crude preparations of phosphoglycolate phosphatase and glycolate oxidase. In our investigations phosphoglycolate formation has been measured, and some enzymatic characteristics of the RuDP oxygenase are compared with the RuDP carboxylase activity of the same protein. Preliminary reports concerning this work have appeared (Lorimer, 1972; Lorimer *et al.*, 1972a).

Experimental Section

Materials

Spinach was grown in a growth chamber as previously described (Andrews *et al.*, 1971). RuDP (tetrasodium salt) was from Sigma Chemical Co., phosphoglycolate from General Biochemicals Inc., and other chemicals and enzymes were of the highest purity commerically available. Purified fraction-1 protein from soybean leaves was supplied by Dr. W. L. Ogren, U. S. Department of Agriculture Regional Soybean Laboratory, Urbana, III.

Methods

Preparation of [U-^{14}C]RuDP. This was prepared in a reaction mixture of 3 ml containing 0.5 mM [U-^{14}C]glucose

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received July 24, 1972.* This work was supported in part by National Science Foundation Grant No. GB 32040X and is published as Journal Article No. 6008 of the Michigan Agricultural Experiment Station.

‡ Present address: Department of Environmental Biology, Research School of Biological Sciences, Australian National University, Canberra City, A.C.T. 2601, Australia.

§ Present address: Vennesland Forschungsstelle der Max Planck Gesellschaft, 1 Berlin 33, Harnackstrasse 23, West Germany.

[1] Abbreviations used are: RuDP, D-ribulose 1,5-diphosphate; Ammediol, 2-amino-2-ethyl-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid.