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In Vitro Assembly of *Neurospora* Assimilatory Nitrate Reductase from Protein Subunits of a *Neurospora* Mutant and the Xanthine Oxidizing or Aldehyde Oxidase Systems of Higher Animals*

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Abstract. *In vitro* assembly or complementation of a hybrid assimilatory nitrate reductase was attained by mixing a preparation of nitrate-induced *N. crassa* mutant *nit-1* specifically with acid-treated (pH 2.5) bovine milk or intestinal xanthine oxidase, rabbit liver aldehyde oxidase, or chicken liver xanthine dehydrogenase. The complementation reaction specifically required induced *nit-1*, the only nitrate reductase mutant of *Neurospora* that lacked xanthine dehydrogenase and was unable to use hypoxathine or nitrate as a sole nitrogen source. The complementing activities of the above acid-treated enzymes correspond to their xanthine or aldehyde oxidizing activity profiles on sucrose density gradients. The resulting soluble, reduced nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate reductases are the same as the *Neurospora* wild type enzyme in sucrose density gradient profile, molecular weight, substrate affinities, and sensitivity to inhibitors and temperature. By analogy to a similar *in vitro* complementation of nitrate reductase in mixtures of induced *nit-1* and individual nonallelic *Neurospora* mutants, or uninduced wild type, the complemented nitrate reductase apparently consists of an inducible protein subunit (possessing inducible NADPH-cytochrome *c* reductase) furnished by *nit-1* and a subunit from the acid-treated xanthine or aldehyde oxidizing system which can substitute for the constitutive component furnished by the other mutants or uninduced wild type. The data suggest that *Neurospora* nitrate reductase and the xanthine oxidizing system and aldehyde oxidase of animals, all of which are molybdenum-containing enzymes catalyzing the reduction of nitrate to nitrite, share a highly similar protein subunit.

Neurospora crassa assimilatory NADPH-nitrate reductase (NADPH: nitrate oxidoreductase, E.C. 1.6.6.2) is a soluble, sulfhydryl protein, with flavine adenine dinucleotide (FAD), cytochrome b_{557} (*N. crassa*), molybdenum, and an unidentified second metal component as prosthetic groups.[1–6] It has a molecular weight of 230,000[6,7] and displays several other inducible enzymic activities including FAD-dependent NADPH-cytochrome *c* reductase,[8] reduced FAD ($FADH_2$)-nitrate reductase, and reduced methylviologen (MVH)-nitrate reductase.[6] *In vitro* complementation of assimilatory NADPH-nitrate reductase by mixing ex-

tracts of certain nonallelic *N. crassa* mutants (nitrate-induced *nit-1* with *nit-2*, *nit-3*, or uninduced wild type) syggested that the enzyme is a heteromultimer formed from the interaction of at least two protein subunits coded for by different cistrons.[7] One subunit(s), uniquely possessed by *nit-1*, is induced by nitrate and is responsible for the early part of the nitrate reductase sequence as reflected by its inducible NADPH-cytochrome *c* reductase activity. The second component(s) representing the latter part of the pathway is a particulate constitutive protein present in all strains (except *nit-1*). It is activated (as indicated by the appearance of $FADH_2$- and MVH-nitrate reductase activities) and solubilized upon combination with the inducible subunit(s).[7]

The present report describes a comparable *in vitro* complementation or assembly of hybrid assimilatory NADPH-nitrate reductase using preparations of induced *Neurospora nit-1* specifically as the source of the inducible protein subunit and *acid-treated* mammalian xanthine oxidase, liver aldehyde oxidase, or avian xanthine dehydrogenase in place of the usual constitutive component. A preliminary account of the present work has appeared.[9]

Materials and Methods. *N. crassa* wild type and nitrate reductase mutant strains were maintained, grown, and *crude extracts* prepared as in the earlier study.[7] Sources of substrates, cofactors, and other chemicals have been cited.[7] NADPH-nitrate reductase, $FADH_2$-nitrate reductase, MVH-nitrate reductase, and NADPH-cytochrome *c* reductase activities were determined and activity units calculated as described.[6] Complementation is expressed in units of NADPH-nitrate reductase activity formed. Xanthine oxidase fractions of various degrees of purity were obtained from several sources[10a] and assayed as described by Nelson and Handler.[10b] The difference in extinction coefficients at 295 nm between xanthine and uric acid of 9.6×10^3 cm^{-1} M^{-1} was used for conversion of the observed absorbance changes into international units of activty,[11-13] i.e., micromoles of xanthine oxidized per minute. Chicken liver xanthine dehydrogenase was purified and expressed in the activity units (rate of reduction of NAD in absorbance units per minute at 340 nm) as described.[14] Xanthine dehydrogenase activity in *Neurospora* extracts was determined in a Turner model 111 fluorometer by an assay based upon the oxidation of 2-amino-4-hydroxypteridine by NAD to the fluorescent product isoxanthopterin.[15] Rabbit liver aldehyde oxidase was purified and expressed in the activity units (rate of oxidation of *N*-methylnicotinamide in absorbance units per minute at 300 nm) described.[16] Acid treatment of the various enzymes was achieved by appropriate dilution of the enzyme in 0.1 N NaCl adjusted to the desired pH with 1 N HCl. Procedures for sucrose gradient sedimentation analyses (SW 39L rotor, 39,000 rpm, 20 hr) protein determination, Stokes radius estimation by Sephadex G-200 gel filtration, and molecular weight calculation were the same as detailed elsewhere[6,7] unless otherwise noted.

Results. Mammalian xanthine oxidase, liver aldehyde oxidase, and chicken liver xanthine dehydrogenase resemble *Neurospora* assimilatory nitrate-reductase in certain respects: each is a molybdenum-containing enzyme capable of catalyzing the reduction of nitrate to nitrite.[17,18] When assayed for the enzymic activities associated with the wild type *Neurospora* assimilatory NADPH-nitrate reductase,[6,7] the above xanthine-oxidizing systems exhibited appreciable MVH-nitrate reductase and to a lesser extent $FADH_2$-nitrate reductase activities (e.g., see Fig. 1). Liver aldehyde oxidase showed, at best, only a trace of MVH-nitrate and no detectable $FADH_2$-nitrate reductase. None possessed NADPH-nitrate reductase or FAD-dependent NADPH-cytochrome *c* reductase activities.

In addition, preliminary observations indicated that of the *Neurospora* nitrate reductase mutants (*nit-1*, *nit-2*, *nit-3*, and *nit-25376*) and wild type tested, only *nit-1* lacked xanthine dehydrogenase and was unable to grow on hypoxanthine or nitrate as the sole nitrogen source similar to certain *Aspergillus* mutants.[19] The latter findings led Pateman *et al.*[19] to postulate a "common cofactor" for nitrate reductase and xanthine dehydrogenase. For these reasons, it seemed feasible to determine if an extract of induced *nit-1* (uniquely possessing inducible NADPH-cytochrome *c* reductase) would complement with the above enzymes to form NADPH-nitrate reductase. Accordingly, mixtures of nitrate-induced *nit-1* extract and partially purified preparations at pH 7.3 of bovine milk xanthine oxidase were incubated in various proportions at room temperature for time intervals up to several hours. All failed to give NADPH-nitrate reductase activity.

Acid solutions (about pH 2) dissociate some proteins,[20-22] including xanthine and liver aldehyde oxidase,[13] into polypeptide subunits. Addition of an extract of induced *nit-1* to acid-inactivated wild type *Neurospora* nitrate reductase significantly restored its NADPH-nitrate reductase and associated activities.[7] These observations prompted experimental attempts at complementation utilizing *acid-treated* xanthine oxidase, xanthine dehydrogenase, or aldehyde oxidase with induced *nit-1*. Figure 1 shows that exposure of xanthine oxidase for 15 min to various pH's ranging from 4.0 to 9.0 failed to generate complementing ability or to affect its enzymic activities. At pH 2.0–3.0, however, appreciable complementing activity appeared, with the enzyme at pH 3.0 still maintaining its full xanthine oxidase, MVH-nitrate, and $FADH_2$-nitrate reductase activities. However, at pH 2.5 (where maximal complementing activity was produced) and lower pH's there was a complete loss of xanthine oxidase and associated activities (Fig. 1) that were not reactivated by subsequent neutralization. A similar pH effect on the appearance of complementing ability and loss of aldehyde oxidase activity was observed for the rabbit liver aldehyde oxidase. The optimal pH treatment of xanthine oxidase for generating complementing

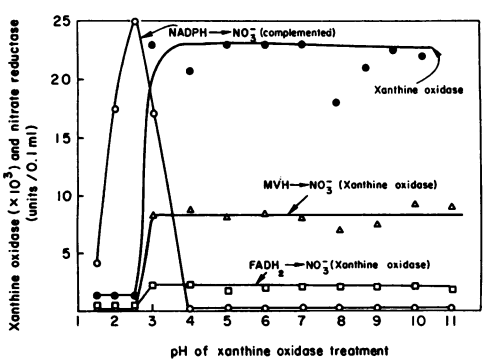

FIG. 1.—Effect of pH on the appearance of complementing activity and on the xanthine oxidase, MVH-, and $FADH_2$-nitrate reductase activities of xanthine oxidase. Bovine milk xanthine oxidase (Univ. of Michigan source) in 0.05 M potassium phosphate, pH 7.8, was diluted 75-fold in a 0.1 N NaCl solution, previously adjusted to the indicated pH's with HCl or NaOH and maintained at 0°C for 15 min before assaying for xanthine oxidase, MVH-, and $FADH_2$-nitrate reductase activities. Complementing activity, which was determined after incubating each of the pH-diluted xanthine oxidase preparations with an equal volume of crude extract of induced *nit-1* (about 10 mg protein/ml) for 15 min at room temperature (final pH of complementation mixture was 6.8) by assaying three different aliquots (0.05, 0.10 and 0.20 ml) of each complementation mixture for NADPH-nitrate reductase activity, is expressed in NADPH-nitrate reductase units formed per 0.1 ml of pH-diluted xanthine oxidase solution.

ability (with *nit-1* to form NADPH-nitrate reductase) is 2.5. By contrast the optimal pH for the complementation reaction is 6.8 (not shown) which is the final pH attained when the acid-treated xanthine oxidase is mixed with induced *nit-1* extract.

Of some 20 different partially purified enzymes[26] at pH 7.3 or adjusted to pH 2.5, none were able to serve in place of the above acid-treated xanthine oxidizing or aldehyde oxidase systems in the complementation reaction. Besides a suitably acid-treated xanthine oxidase, xanthine dehydrogenase, or aldehyde oxidase, the complementation reaction has a specific requirement for the nitrate-induced *nit-1* preparation. Extracts from either uninduced *nit-1*, uninduced wild type, or any of the other *nit* mutants (induced or uninduced) failed to substitute for the induced *nit-1* extract (not shown). On rare occasions aged xanthine oxidase preparations, particularly when stored in 0.6 saturated ammonium sulfate, showed some complementing ability without the usual prior acid treatment.

Direct involvement of xanthine oxidase in complementation is further implicated by the following observations. The sucrose density gradient profile of bovine milk xanthine oxidase (sedimentation coefficient, $s_{20,w}$ = 11.1 S) and its associated MVH-nitrate and $FADH_2$-nitrate reductase activities, coincides with that of its complementing activity (generated by subsequent acidification to pH 2.5, Fig. 2*A*). The same results were obtained with the other bovine milk and intestinal xanthine oxidase preparations, chicken liver xanthine dehydrogenase, and rabbit liver aldehyde oxidase. The faster sedimenting minor peak of xanthine oxidase ($s_{20,w}$ = 15 S) also contains complementing ability. This peak, absent from most of the other preparations examined, is probably the enzymatically active dimer of xanthine oxidase observed by others.[10,23] The complementing activity of pH 2.5-treated xanthine oxidase on an acid sucrose density gradient yields an $s_{20,w}$ of about 6 S for the active complementing species (Fig. 2*B*). Dissociation of bovine milk xanthine oxidase ($s_{20,w}$ 11.7 S, mol wt 300,000) and rabbit liver aldehyde oxidase ($s_{20,w}$ 11.5 S, mol wt 275,000) by Nelson and Handler[10] into molecules of about half the size was effected by the same acid treatment.

The sucrose density gradient profile of complemented NADPH-nitrate reductase formed in a mixture of induced *nit-1* and acid-treated xanthine oxidase (Fig. 2*C*) was similar to those of the wild type enzyme and of the enzyme formed by *in vitro* complementation in *Neurospora* extracts.[7] It exhibits a single peak ($s_{20,w}$ = 7.8 S) that includes inducible cytochrome *c* reductase and MVH- and $FADH_2$-nitrate reductase activities, but differs in the ratio of associated enzymic activities. As in the earlier work,[7] not all of the slower sedimenting inducible cytochrome *c* reductase ($s_{20,w}$ = 4.5 S) contributed by the *nit-1* fraction (Fig. 2*C*) is utilized in the complementation reaction with acidified xanthine oxidase to yield the faster-sedimenting form associated with nitrate reductase, since some is still evident in the sucrose density profile (Fig. 2*C*). NADPH-nitrate reductases formed by complementation of induced *nit-1* and acid-treated bovine milk xanthine oxidase, bovine intestinal xanthine oxidase, chicken liver xanthine dehydrogenase, or rabbit liver aldehyde oxidase exhibit sucrose density gradient profiles similar to that shown in Figure 2*C*. The NADPH-nitrate reductases

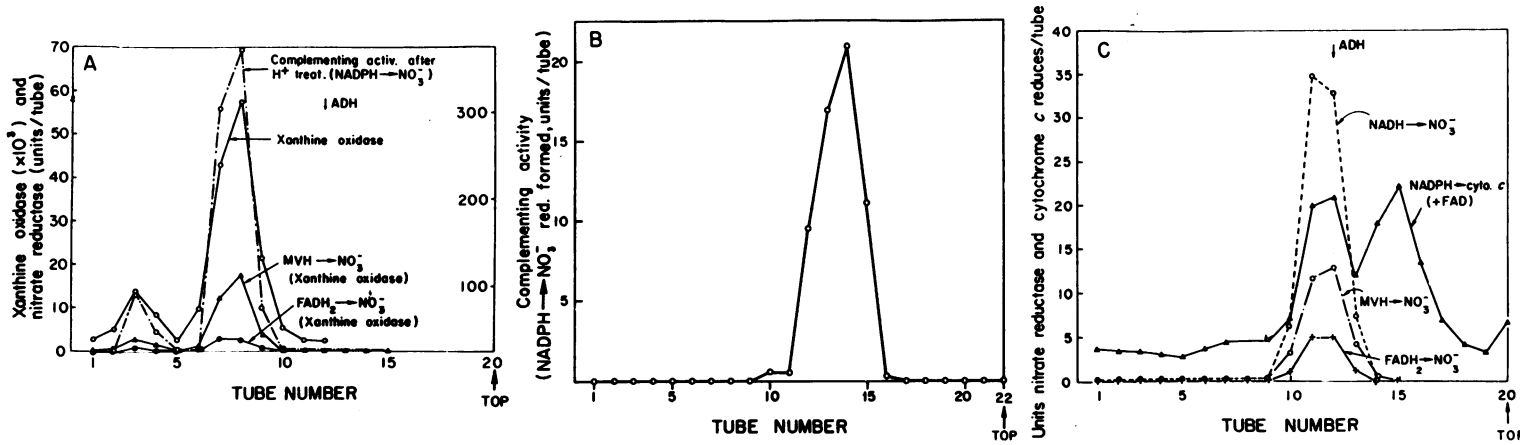

FIG. 2.—Sucrose density gradient profiles of (*A*) xanthine oxidase, its associated MVH- and $FADH_2$-nitrate reductase activities, and its complementing activity after acid treatment, (*B*) acid-treated xanthine oxidase is monitored by its complementing activity, and (*C*) partially purified complemented nitrate reductase and its associated activities formed by the interaction of acid-treated xanthine oxidase and an ammonium sulfate fraction of induced *nit-1*.

(*A*) 0.2 ml of bovine milk xanthine oxidase (Univ. of Michigan) was layered on a linear sucrose gradient (in 0.05 M potassium phosphate, pH 8.0) and the collected fractions assayed for xanthine oxidase and associated nitrate reductase activities. For complementing activity 0.01 ml from each tube was diluted 10-fold in 0.1 N NaCl, pH 1.9 incubated with an equal volume of crude extract of induced *nit-1* (about 10 mg protein/ml) for 15 min at room temperature, and assayed for NADPH-nitrate reductase for 30 min. Yeast alcohol dehydrogenase (ADH), $s_{20,w}$ = 7.4 S, was used as an internal standard. Xanthine oxidase recovery from the sucrose density gradient was about 35%.

(*B*) 0.2 ml of bovine milk xanthine oxidase (Duke Univ.) adjusted to pH 2.5 was layered on a linear 4.6 ml sucrose gradient (10–25%, w/v in 0.1 N NaCl adjusted with HCl to pH 2.5) and centrifuged at 39,000 rpm in the usual manner for 16 hr 20 min. Complementing activity was determined by incubating 0.1 ml from each tube with 0.2 ml crude extract of induced *nit-1* for 30 min at room temperature and assaying for NADPH-nitrate reductase for 1 hr. ADH was used as an external standard in the same experiment on a similar sucrose linear gradient but in 0.05 M potassium phosphate, pH 8.0. Complementing activity recovery was 35% compared to that attained with xanthine oxidase acidified after a similar sucrose density gradient procedure (at pH 8.0).

(*C*) bovine milk xanthine oxidase (Univ. of Michigan) was acidified by 50-fold dilution in 0.1 N NaCl, pH 2.5 and complemented with a partially purified fraction of induced *nit-1* crude extract. The complemented NADPH-nitrate reductase was partially purified and a sample containing 201 unite (spec. act. 183) subjected to the usual sucrose density gradient procedures with ADH as an internal standard. The contents of each tube were assayed for NADPH-nitrate reductase and associated activities. Recovery of the complemented enzyme from the sucrose density gradient was 50%.

formed by complementation with the above acid-treated xanthine and aldehyde oxidizing systems were shown to have a sedimentation coefficient in all cases of 7.8 S, a Stokes radius ranging from 70 to 72 Å, and thus a calculated molecular weight of 232,000–238,000. The corresponding values determined for the wild type *Neurospora* enzyme were 7.9 S, 70 Å, and 235,000.

The complemented NADPH-nitrate reductases prepared by interaction of *nit-1* extract and the xanthine oxidases obtained from the University of Michigan and Duke University were partially purified and more extensively characterized. They proved to be similar to the wild type enzyme in their substrate and cofactor affinities, pH optima, sensitivity to metal binding agents and -SH inhibitors, and response to 50°C temperature.[6] The complemented nitrate reductase originating from the Duke University xanthine oxidase was concentrated and shown to contain cytochrome $b_{557(N.\ crassa)}$ that was enzymatically reduced by NADPH and added FAD.[5]

The time course of NADPH-nitrate reductase formation at 23°C by the interaction of acid-treated xanthine oxidase and induced *nit-1* extract showed a rapid initial rate (about 10 times that at 0°C thus indicating the temperature-dependent nature of the reaction) that leveled off after 20–30 min, with little or no change in the remaining 90 min. The active complementing component(s) produced by acid treatment of xanthine oxidase displays a marked lability. It undergoes a 60–75% loss in complementing ability after 1 hr at 0°C, compared to a 75% loss after only 15 min at 23°C. Subsequently neutralized xanthine oxidase (after 5–10 min at pH 2.5) exhibited the same time course of lability as the original acid-treated enzyme from which it was derived. The quantity of NADPH-nitrate reductase formed by complementation is approximately proportional to increasing levels of acidified xanthine oxidase over a certain concentration range using a constant amount of induced *nit-1* extract, and to increasing concentrations of induced *nit-1* extract using a constant level of acidified xanthine oxidase (not shown).

Discussion. The assembly of assimilatory NADPH-nitrate reductase by interaction of induced *nit-1* extract with the acid-treated xanthine oxidizing or aldehyde oxidase systems of higher animals and that attained by mixing the *nit-1* extract with preparations of certain non-allelic *Neurospora* mutants, uninduced wild type, or acid-treated wild type *Neurospora* nitrate reductase[7] share several basic features: (*a*) a specific requirement for an extract of nitrate-induced *nit-1;* (*b*) a reconstitution or appearance of MVH- and $FADH_2$-nitrate reductase activities typically associated with NADPH-nitrate reductase; (*c*) a conversion of inducible NADPH-cytochrome *c* reductase ($s_{20,w}$ = 4.5 S) to a faster sedimenting form (Fig. 2*A*) associated with NADPH-nitrate reductase ($s_{20,w}$ = 7.9 S) suggestive of subunit assembly; and (*d*) a requirement for prior acid treatment (known to dissociate some proteins into polypeptide subunits[20–22]) in the case of the wild type *Neurospora* nitrate reductase,[7] xanthine oxidase, xanthine dehydrogenase, or aldehyde oxidase in order that complementation might occur.

The above characteristics support the hypothesis that *in vitro* complementation of assimilatory NADPH-nitrate reductase is the result of the interaction of at

least two dissimilar protein subunits coded for by different cistrons.[7] One is the nitrate-inducible component(s) of *nit-1* ($s_{20,w}$ of 4.5 S) responsible for the early part of the electron transport chain as reflected by inducible NADPH-cytochrome *c* reductase. The second subunit(s) representing the latter part of the electron transport pathway can be furnished by two general sources: either in a form that exhibits no MVH-nitrate or $FADH_2$-nitrate reductase activity and requires no prior acid treatment for complementing activity (from *nit-2* and uninduced wild type[7]), or in a form displaying MVH- or $FADH_2$-nitrate reductase, that must first be dissociated (by acid treatment of wild type *Neurospora* nitrate reductase, or the xanthine oxidizing or aldehyde oxidase systems of higher animals) in order to acquire complementing ability. Complementation, or the assembly of protein subunits into a larger enzyme complex, is accompanied by reactivation of MVH- and $FADH_2$-nitrate reductases, often to relatively high values. The present work points to *nit-1* as the source of cytochrome b_{557} since the purified xanthine and aldehyde oxidizing systems lack cytochromes.

The finding that subunits of certain molybdenum-containing enzymes from diverse phylogenetic sources (namely, fungi, birds, and mammals) can substitute for the constitutive component in the *in vitro* complementation reaction with the inducible subunit(s) of *nit-1* to form assimilatory NADPH-nitrate reductase elicits at least two explanations: (*a*) the polypeptides from diverse sources are quite different from one another but have retained regions of similarity that makes them active in complementation, or (*b*) the complementing subunits from different sources are very similar, if not indistinguishable, in all respects, thus accounting for the highly specific phenomena of recognition, interaction, and assembly of protein subunits. The latter interpretation is supported by the report of little or no success in cross hybridization experiments between subunits of different enzymes,[24] particularly in acid dissociation experiments,[25] indicating that the assembly of a multimeric enzyme is highly specific. The striking similarities in the properties of the complemented enzymes and wild type NADPH-nitrate reductase would suggest that the subunits of the xanthine and aldehyde oxidizing systems closely resemble the uninduced constitutive component from *Neurospora*. This viewpoint implies the selection during the course of evolution of a protein subunit involved in electron transport that is shared in common by certain enzymes among a diversity of organisms, as well as in the same organism as indicated by the lack of both nitrate reductase and xanthine dehydrogenase in certain mutants of *Aspergillus*[19] and in *nit-1* of *Neurospora*.

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[10] (*a*) Bovine milk xanthine oxidase fractions of 0.75 units/mg protein and 0.72 units/mg protein were kindly provided by Dr. V. Massey (University of Michigan) and Dr. K. V. Rajagopalan (Duke University), respectively. Xanthine oxidase (0.58 units/mg protein) purified from bovine small intestine was a gift from Dr. G. G. Roussos, Veterans Administration. Bovine milk xanthine oxidase preparations were also obtained from Sigma Biochemicals (grade I, 0.72 units/mg protein; grade II, 0.14 units/mg protein), Worthington Biochemicals (0.46 units/mg protein) and buttermilk xanthine oxidase from Mann Research Laboratories (0.30 units/mg protein). (*b*) Nelson, C. A., and P. Handler, *J. Biol. Chem.*, **243**, 5368 (1968).
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[26] The following stock enzymes, diluted 50-fold in 0.01 M potassium phosphate, pH 7.3, or in 0.1 M NaCl adjusted to pH 2.5, were tested for complementing activity as described in Fig. 1 except that the complementation reaction mixture was incubated for 30 min. From *Mannheim-Boeringer:* enolase (crystalline), muscle lactate dehydrogenase (cryst.), pyruvate kinase (cryst.), glutamate dehydrogenase (cryst.), diaphorase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, muscle fructose-6-phosphate kinase. From *Sigma:* rabbit muscle triose phosphate isomerase (cryst.), bovine pancreas ribonuclease A, rabbit muscle 5′-adenylic acid deaminase, jack bean urease. From *Worthington:* muramidase (cryst.), trypsin (cryst.), ribonuclease (cryst.), yeast lactic acid dehydrogenase. From *Calbiochem:* yeast phosphoglucose isomerase (cryst.), rabbit muscle aldolase, From *Mann:* Bovine pancreas carboxypeptidase A, mixture of bovine serum albumin (cryst.) and 10^{-6} M Na_2MoO_4.