Purification and Properties of Rabbit Muscle L-Glycerol 3-Phosphate Dehydrogenase

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A modified procedure has been developed for the purification of rabbit muscle L-glycerol 3-phosphate dehydrogenase. The product of the preparation satisfies all criteria of homogeneity. Some physical properties of the enzyme have been re-investigated. The results suggest that previous preparations may have been contaminated with significant amounts of heavy-molecular-weight protein.

Glycerol phosphate dehydrogenase (L-glycerol-3-phosphate-NAD⁺ oxidoreductase, EC 1.1.1.8.) has been studied for many years; there is, however, a considerable amount of contradictory information about the enzyme in the literature. For example, Young & Pace (1958) estimated that the molecular weight was 173 000 with a partial specific volume of 0.75 ml/g whereas Van Eys *et al.* (1959) calculated a value of 78 000 with a partial specific volume of 0.70 ml/g. This latter estimation of the molecular weight became the accepted value until Fondy *et al.* (1969) obtained a molecular weight of 60 000 with a partial specific volume of 0.747 ml/g estimated from amino acid analysis.

Similar disagreement exists about the nature of the non-protein component of glycerol phosphate dehydrogenase. Enzyme isolated by several different techniques was found to have a higher extinction at 260 nm than would be expected from a simple protein. It was suggested, therefore, that a non-protein component absorbing around 260 nm was bound to the enzyme. Ankel *et al.* (1960) suggested that the compound was adenosine diphosphate ribose, Van Eys *et al.* (1959) and Van Eys (1960) identified a thiazolylsuccinic acid derivative and Celliers *et al.* (1963) obtained at least two non-protein components of which one was adenosine diphosphate ribose but neither was a thiazolylsuccinic acid derivative. To add to the confusion Ankel *et al.* (1960) and Van Eys *et al.* (1959) found that removal of the component with charcoal had no effect on enzymic activity whereas Telegdi & Keleti (1966) obtained a 100% increase in specific activity on treatment with charcoal to raise the E_{280}/E_{260} ratio from 1.3 to 1.6.

In view of the conflicting statements it was thought necessary to re-investigate these properties of glycerol phosphate dehydrogenase, together with the unconfirmed reports that the enzyme contained

significant amounts of zinc (Vallee *et al.*, 1956) and that it will catalyse the reduction of glyceraldehyde 3-phosphate by NADH (Telegdi & Keleti, 1968; Telegdi, 1968), before commencing a detailed kinetic study. It seemed possible that some of the discrepancies may have resulted from the use of heterogeneous enzyme preparations. We decided therefore to try to introduce chromatographic procedures into the isolation procedure with the object of obtaining a product which would satisfy all criteria of purity before beginning our study.

Materials and Methods

Materials

NAD⁺ (grade II) and NADH (grade I) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., and used without further purification. DL-Glycerol phosphate (sodium salt) and glyceraldehyde 3-phosphate diethylacetal monobarium salt were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Dithizone was the product of BDH Chemicals, Poole, Dorset, U.K. All other chemicals were analytical-reagent grade whenever available, purchased mainly from Fisons Chemicals Ltd., Loughborough, Leics., U.K. Whatman DE-11 DEAE-cellulose was washed with 0.5 M-NaCl–0.5 M-NaOH before being equilibrated to the appropriate pH and ionic strength. New Zealand White rabbits (18 months old) were purchased from L. Moore, Thornton, Bradford, Yorks., U.K. Glass-distilled water was used throughout.

Methods

All dialysis tubing was boiled in 10 mM-EDTA, pH 7.0, and washed well with water before use. Unless otherwise stated all buffers contained 0.3 mM-EDTA. Solutions of glycerol phosphate dehydrogenase were prepared by dialysing crystalline suspensions overnight against sodium phosphate buffers of the appropriate pH and ionic strength.

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Protein concentrations. During the preparation protein concentrations and specific activities were determined spectrophotometrically assuming $E_{1\text{cm}}^{1\%} = 10$ at 280nm. For recrystallized glycerol phosphate dehydrogenase solutions a specific extinction coefficient at 280nm of $E_{1\text{cm}}^{1\%} = 5.15$ based on dry-weight measurements was used.

Enzyme assay. Routine assays were performed at 340nm and 25°C by using a Zeiss PM QII spectrophotometer. The assay mixture was 3ml of a solution containing: DL-glycerol phosphate, 120μmol; NAD⁺, 2.1μmol and glycine buffer, pH10.0, 200μmol. The reaction was initiated by addition of enzyme, a suitable amount being that which caused $\Delta E < 0.2/\text{min}$. One unit of enzyme activity was defined as that amount of enzyme producing 1μmol of NADH/min under these conditions of assay.

Preparation of glycerol phosphate dehydrogenase. A typical flow sheet for the preparation of L-glycerol 3-phosphate dehydrogenase from six rabbits appears in Table 1. The rabbits were killed by a blow on the neck and bled. The muscle (3.6kg) was removed from the hind legs and back and minced through a chilled mincer. All subsequent steps were performed at 4°C. Portions (100g) of minced muscle were blended with 200ml of disodium EDTA solution (0.5g/l) at full speed for 1½min in an MSE Ato-Mix blender. The batches were combined and the mixture stirred manually for 30min before being centrifuged at 2200g for 1h. The supernatant was decanted and stored while the precipitate was blended in 400g portions with 200ml of EDTA, stirred for 15min and centrifuged as before. The two supernatants were then combined and filtered through glass-wool to remove fat. The insoluble muscle material was discarded.

Finely ground (NH₄)₂SO₄ (260g/l of extract) was added over a 1h period. After a further 2h of standing the precipitate was removed by centrifugation at

2200g for 30min and discarded. (NH₄)₂SO₄ (170g/l of extract) was now added over a 1h period and after standing for 1h the precipitate, which contained the glycerol phosphate dehydrogenase activity, was collected by centrifugation at 2200g for 40min. The precipitate was dissolved in a minimum volume of 5mm-sodium phosphate buffer, pH7.0, and dialysed against the same buffer.

After dialysis the protein solution (fraction 1) was centrifuged to remove any insoluble material and then applied to a column (100cm×5cm) of DEAE-cellulose, which had previously been equilibrated with 5mm-sodium phosphate buffer, pH7.0. The column was washed with 5mm-sodium phosphate buffer, pH7.0, until the extinction of the effluent at 280nm was below 0.4, whereupon enzyme was eluted from the column with 50mm-sodium phosphate buffer, pH7.0. The protein concentration of the active effluent was about 5mg/ml. Glycerol phosphate dehydrogenase was precipitated by dialysis against 50mm-sodium phosphate buffer, pH6.2, +2mm-EDTA, which contained sufficient (NH₄)₂SO₄ to bring the total volume, both inside and outside the dialysis bag, to a final concentration of 430g/l. After overnight dialysis the volume inside the dialysis bag had decreased to about one-third of the original and the enzyme precipitated with greater than 95% yield.

The precipitate was dissolved in 50mm-sodium phosphate buffer, pH6.2, to a nominal concentration of 50mg/ml ($E_{1\text{cm}}^{280} = 50$) (fraction 2). Inactive protein was removed by slow addition of 0.43vol. of (NH₄)₂SO₄ solution, saturated at room temperature; after 15min the precipitate was removed and discarded. The enzyme was then precipitated by addition of a further 0.57vol. of (NH₄)₂SO₄ solution, saturated at room temperature. The precipitate was collected by centrifugation, dissolved in a minimum

Table 1. *Purification of glycerol 3-phosphate dehydrogenase*

Step	Volume (ml)	Total protein (mg)	Total units	Sp. activity	Yield (%)	Purification
Crude extract	6900	182000	78000	0.43	100	1
First (NH ₄) ₂ SO ₄ fraction after dialysis (fraction 1)	820	49500	58000	1.17	74	27
Combined eluate from DEAE-cellulose column after concentration (fraction 2)	21	1050	38000	35	49	81
Second (NH ₄) ₂ SO ₄ fraction after dialysis (fraction 3)	7	525	31000	58	40	134
Combined eluate from Sephadex G-200 concentration (fraction 4)	4.3	230	22000	95	28	220
Crystallized and twice recrystallized	3.3	165 320*	19800	119 62*	25	245

* Calculated by using protein concentration determined by dry-weight measurements.

volume of 100mM-sodium phosphate buffer, pH 6.2, and dialysed against the same buffer. After dialysis portions of about 4ml of the solution (fraction 3) were applied to a column (100cm $\times$ 2.5cm) of Sephadex G-200 that had previously been equilibrated with 100mM-sodium phosphate buffer, pH 6.2. (It was necessary to use two or three columns of this size to accommodate all the preparation.) The glycerol phosphate dehydrogenase was eluted from the Sephadex column at a concentration of about 3mg/ml and precipitated by dialysis against $(\text{NH}_4)_2\text{SO}_4$ as before. The precipitate was dissolved in a minimum volume of 50mM-sodium phosphate, pH 6.2 (fraction 4), and saturated $(\text{NH}_4)_2\text{SO}_4$ solution was then added very slowly until the protein solution developed a faint opalescence, whereupon it was left for 15min to allow glycerol phosphate dehydrogenase to crystallize. Crystallization was completed by slowly adding an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution to that required to cause the opalescence. The crystals were collected by centrifugation, dissolved in a minimum volume of 50mM-sodium phosphate buffer, pH 6.2, and recrystallized twice in the same manner.

The Sephadex G-200 step was omitted from some early preparations and the glycerol phosphate dehydrogenase crystallized, in the manner described above, from the DEAE-cellulose column effluent after it had been concentrated with $(\text{NH}_4)_2\text{SO}_4$ (fraction 3). In these cases the maximum apparent specific activity obtained was about 30% less than that obtained by including the Sephadex G-200 step. When extinction coefficients based on dry-weight measurements are used this indicates a true amount of about 10% impurity.

Zinc estimations. Samples containing known amounts of glycerol phosphate dehydrogenase were dried slowly at 80°C and dry-ashed at 500°C. The ash was dissolved in 6M-HCl (microanalytical grade), dried and ashed as before. The ash was again dissolved in 6M-HCl and the zinc content of the solution estimated by the dithizone method of Cremona & Singer (1964).

All solutions used in these estimations were rendered zinc-free by shaking with dithizone and stored in polyethylene bottles. All glassware and storage bottles were washed in 10% (v/v) HNO_3 .

Molecular-weight determinations. Short-column sedimentation-equilibrium experiments were performed by the methods of Van Holde & Baldwin (1958) or Yphantis (1964) at 10°C with enzyme solutions in 100mM-sodium phosphate buffer, pH 6.2, in an MSE analytical ultracentrifuge. Experiments by the method of Van Holde & Baldwin (1958) were performed by using schlieren optics with rotor speeds of about 9000rev./min and enzyme concentrations of about 3mg/ml. For the Yphantis (1964) method interference optics were used with rotor

speeds of 16000–20000rev./min and enzyme concentrations of 0.4mg/ml. For both types of experiment a 10mm double-sector cell was used with the enzyme in one compartment and a sample of the buffer in the other. Glycerol phosphate dehydrogenase was completely stable at 10°C for the duration of the experiments.

The partial specific volume was determined at 25°C with a pycnometer as described by Schachman (1957). The value at 10°C was estimated by the method of Edelstein & Schachman (1967) in which sedimentation-equilibrium experiments with meniscus depletion were performed on solutions of enzyme in buffer solutions prepared in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. The results were calculated as described by Edelstein & Schachman (1967).

Coenzyme binding. The binding of NADH to glycerol phosphate dehydrogenase was monitored spectrophotometrically and fluorimetrically. Fluorimetric titrations were performed in a recording filter fluorimeter of the type described by Dalziel (1962) at 25°C in sodium phosphate buffer, pH 6.0, $I = 0.1$.

Spectrophotometric titrations were performed in a Zeiss PM QII spectrophotometer at 340nm in sodium phosphate buffer, pH 6.0, $I = 0.1$, at 25°C. For both types of experiment the enzyme was stable for the period of the titration and no blank oxidation of NADH was detected.

The spectrum of the enzyme-bound NADH was obtained in a Cary 14 UV recording spectrophotometer.

Dry-weight measurement. A solution of glycerol phosphate dehydrogenase in 5mM-sodium phosphate buffer, pH 6.2, was centrifuged at 40000g for 20min to remove any debris. The $E_{1\text{cm}}^{280}$ of the solution was determined and samples of the enzyme (containing about 20mg) and the buffer were pipetted into pre-weighed vials. The samples were dried slowly at 80°C and maintained at 80°C under vacuum over CaCl_2 until at constant weight.

Results and Discussion

We have developed a new procedure for the purification of glycerol phosphate dehydrogenase from rabbit muscle. The main difference from other existing procedures is the inclusion of chromatography on DEAE-cellulose. Such chromatography has not been used in any previous glycerol phosphate dehydrogenase preparation. Van Eys *et al.* (1959) did report that a DEAE-cellulose column may be included in the preparation, but were unable to demonstrate any binding of the enzyme to the cellulose at pH values below 8.0. Under our conditions at pH 7.0, however, glycerol phosphate dehydrogenase was bound to the column, and most of the other protein passed straight through. The puri-

fication of about 30-fold with a yield of 65% obtained on elution of the enzyme (see Table 1) makes DEAE-cellulose chromatography a very powerful technique for the purification of this enzyme. A Sephadex G-200 step was also included to remove high-molecular-weight proteins that could not be totally separated from glycerol phosphate dehydrogenase by repeated crystallization. The introduction of these two useful column steps into what is otherwise a very similar procedure to that of Beisenherz *et al.* (1955) means that the enzyme is crystallized from a solution of much higher specific activity. This provides conditions favourable to the preparation of a better product. As discussed below the specific activity was significantly higher than those obtained by the method of Beisenherz *et al.* (1955).

Glycerol phosphate dehydrogenase prepared by direct crystallization of the $(\text{NH}_4)_2\text{SO}_4$ fraction after the DEAE-cellulose column (fraction 3, Table 1), omitting the Sephadex G-200 chromatography, has a maximum apparent specific activity of 85 units/mg instead of 120 units/mg obtained by inclusion of the Sephadex G-200 stage. The product of this earlier preparation appeared to be homogeneous when analysed by polyacrylamide-gel electrophoresis by the

method of Clarke (1964) at pH 8.3 in 38 mM-glycine–5 mM-Tris buffer and at pH 6.6 in 18 mM-glycine–33 mM-2,6-lutidine buffer. Sedimentation-velocity studies showed a single sedimenting boundary. However, analysis of sedimentation-equilibrium studies in 3 mm columns by the method of Van Holde & Baldwin (1958) led to markedly curved plots of the type shown in Fig. 1. The pronounced upward curvature of the plots towards the bottom of the cell suggested that the preparation was heterogeneous, containing some high-molecular-weight proteins. Similarly curved LaBar plots have been reported by Fondy *et al.* (1969) and they have suggested that the curvature could be a consequence of aggregation of glycerol phosphate dehydrogenase at high protein concentrations or precipitation of denatured protein.

Sedimentation-equilibrium experiments on glycerol phosphate dehydrogenase of a higher specific activity, obtained by using the Sephadex G-200 column, gave plots which were clearly linear throughout the entire length of the column, as shown in Fig. 1. This suggests that the curvature obtained previously was the result of a heterogeneous preparation and not a consequence of protein aggregation or denaturation occurring in the ultracentrifuge. It is interesting that with these preparations sedimentation-equilibrium experiments of this type should prove a more sensitive test of homogeneity than either polyacrylamide-gel electrophoresis or sedimentation-velocity studies.

The absorption spectrum of glycerol phosphate dehydrogenase isolated by our procedure has a maximum absorption at 280 nm and a specific extinction coefficient at 280 nm determined from dry-weight measurements of $E_{1\text{cm}}^{1\%} = 5.15$, which is somewhat lower than the value used by Holbrook *et al.* (1972) and a lot lower than other quoted values (see Fondy *et al.*, 1969). When this extinction coefficient is used to determine protein concentrations the maximum specific activity usually obtained was 62 units/mg when assayed under the conditions described above. This is equivalent to 6200 units/g per litre when assayed as described by Beisenherz *et al.* (1955), which is much higher than the usual specific activity of 4500 units/g per litre and significantly higher than the maximum value reported by these workers of 5700 units/g per litre.

The specific extinction coefficient at 280 nm of glycerol phosphate dehydrogenase of apparent specific activity 75 units/mg isolated without the use of the Sephadex G-200 column was estimated to be $E_{1\text{cm}}^{1\%} = 6.9$ from dry-weight measurements, which is very similar to the values quoted by Fondy *et al.* (1969). This suggests that these preparations contained contaminating protein with a relatively high extinction at 280 nm and strengthens the conclusion from the ultracentrifuge results that the preparations were impure.

The E_{280}/E_{260} ratio of our preparation is 1.62,

Fig. 1. Plot of data from sedimentation-equilibrium experiments by the method of Van Holde & Baldwin (1958) with enzyme preparations isolated with (○) and without (●) the use of Sephadex G-200 chromatography

The experiments were performed at 10°C in 0.1M-sodium phosphate buffer, pH 6.2, with rotor speeds of 8550 rev./min (○) and 9000 rev./min (●).

which is higher than that of other preparations. Thus the absorption spectrum of the enzyme does not show the high relative 260nm absorption common to other preparations, which is usually attributed to the non-protein component. Treatment with charcoal washed with 2M-HCl and 1mM-EDTA, pH 7.0, had no effect on either the enzymic activity or the E_{280}/E_{260} ratio. This suggests that glycerol phosphate dehydrogenase isolated by our procedure does not contain any non-protein components. Free adenosine diphosphate ribose is bound rather firmly to DEAE-cellulose (Dalziel, 1963) and it is possible that any non-specifically associated adenosine diphosphate ribose (or similar compound) would be removed during the DEAE-cellulose chromatography.

Careful analysis of the elemental zinc content of glycerol phosphate dehydrogenase shows a maximum possible value of 0.08 atom/molecule. This obviously is of no structural or catalytic importance. The previous report that glycerol phosphate dehydrogenase

contained significant amounts of zinc (Vallee *et al.*, 1956) probably arose from impurities in the enzyme preparations used.

We were unable to demonstrate any catalysis of the reduction of glyceraldehyde 3-phosphate by NADH, even at high glycerol phosphate dehydrogenase concentrations, under conditions that should have been very favourable (Telegdi & Keleti, 1968; Telegdi, 1968). We conclude therefore that highly purified glycerol phosphate dehydrogenase does not catalyse this reaction. It seems probable that the enzyme preparation used by Telegdi & Keleti (1968) contained some triose phosphate isomerase.

The binding of NADH to glycerol phosphate dehydrogenase is accompanied by marked changes in the NADH absorption spectrum, which have been studied by Fisher *et al.* (1969) who have shown that the NADH difference spectrum above 300nm may be resolved into a 40% hypochromicity with a red shift of 30nm in the position of the absorption maximum. The NADH difference spectrum which we measured was very similar to that shown by Fisher *et al.* (1969). However, the absorption spectrum of the enzyme-bound NADH measured directly (Fig. 2) gave no evidence of any shift in the position of the absorption maximum. The disturbances in this spectrum at 290nm are probably a consequence of perturbation of tryptophan molecules in the enzyme by the enzyme-bound NADH.

The hypochromic affect at 340nm allows the binding of NADH to the enzyme to be studied spectrophotometrically. At pH6.0 NADH binds very tightly to glycerol phosphate dehydrogenase (the dissociation constant for the enzyme-NADH complex is $0.02\mu\text{M}$) and titrations of glycerol phosphate dehydrogenase with NADH at this pH may

Fig. 2. Absorption spectrum of NADH bound to glycerol phosphate dehydrogenase

The extinction of a mixture of $100\mu\text{M}$ -NADH and $200\mu\text{M}$ -glycerol 3-phosphate dehydrogenase in sodium phosphate buffer, pH6.0, $I=0.1$, was read against a similar solution of enzyme. Molar extinction coefficients for the spectrum were calculated by using $\epsilon_{340} = 3.75 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, which was estimated from spectrophotometric titrations. No allowance is required for the extinction of free NADH, since at pH6.0 the dissociation constant of the enzyme-NADH complex is $0.02\mu\text{M}$ (P. Bentley & F. M. Dickinson, unpublished results) and the amount of free NADH under these conditions will be insignificant.

Fig. 3. Spectrophotometric and fluorimetric titrations of glycerol phosphate dehydrogenase with NADH in sodium phosphate buffer, pH6.0, at 25°C

Spectrophotometric titrations (○) were done with 2.2mg of enzyme/ml, and fluorimetric titrations (●) with $92.3\mu\text{g}$ of enzyme/ml.

be used to determine the concentration of active sites. Fig. 3 shows the results of one such titration in sodium phosphate buffer, pH 6.0, $I = 0.1$, at 25°C measured spectrophotometrically at 340 nm. The point at which the enzyme becomes saturated and free NADH appears in solution is clearly shown by the sharp increase in the gradient of the plot. The minimum molecular weight calculated from this titration is 37800. The initial slope of Fig. 3 suggests a value for the molar extinction coefficient of the enzyme-NADH complex, $\epsilon_{340} = 3.75 \times 10^3$ litre·mol⁻¹·cm⁻¹. The value of this extinction coefficient did not appear to vary over a pH range from 6 to 9.

The binding of NADH to glycerol phosphate dehydrogenase is also accompanied by a large fluorescence enhancement, which may be used to study the NADH binding. Fig. 3 also shows the results of a fluorimetric titration of glycerol phosphate dehydrogenase with NADH in sodium phosphate buffer, pH 6.0, $I = 0.1$, at 25°C. The point at which the enzyme becomes saturated is clearly shown by the sharp decrease in the slope of the plot. The minimum molecular weight calculated from the titration is 39300. The difference between this value and that reported by Holbrook *et al.* (1972) probably arises from the different values used for the specific extinction coefficient of the enzyme at 280 nm as discussed above.

The partial specific volume of the enzyme was determined pycnometrically at 25°C and a value of 0.743 ± 0.005 ml/g obtained. This agrees well with that calculated from the amino acid analysis (Fondy *et al.*, 1969). A value obtained at 10°C by the method of Edelstein & Schachman (1967) involving sedimentation-equilibrium experiments on solutions of enzyme in ¹H₂O and ²H₂O was 0.730 ml/g. The difference between these two values is probably a reflexion of the effect of temperature on the partial specific volume and indicates a temperature dependence of 0.87 μ l/g per °C, which is similar to that measured in some other proteins (Reithel & Sakura, 1963).

When a value of the partial specific volume of 0.730 ml/g is used in conjunction with data (Fig. 1) from sedimentation-equilibrium experiments at 10°C in the manner of Van Holde & Baldwin (1958), a value of 76000 is obtained for the molecular weight. Several short-column sedimentation-equilibrium experiments by the method of Yphantis (1964) also gave very similar values. A molecular weight of 76000 agrees with the estimations of Van Eys *et al.* (1959) and Brosemer & Kuhn (1969) but is quite inconsistent with the low value of 60000 obtained by Fondy *et al.* (1969). Note that the apparent agreement with the molecular weight estimated by Van Eys *et al.* (1959) is fortuitous, because these authors used a value of 0.70 ml/g for the partial specific volume. If this value is corrected a molecular

weight of about 90000 is obtained, which suggests that the enzyme preparation used may have been contaminated with high-molecular-weight protein.

The results of the titrations of glycerol phosphate dehydrogenase with NADH also support a molecular weight of 76000. Spectrophotometric titrations suggest that two molecules of NADH are bound per 75600 g of protein whereas the fluorometric titrations suggest that two molecules of NADH are bound per 78600 g of protein. Of the two values, that obtained from the spectrophotometric titration is probably the more accurate because the high concentrations of enzyme used ensure lower concentrations of free NADH at the point at which the enzyme becomes saturated.

One important conclusion of this investigation is that commonly used glycerol phosphate dehydrogenase preparations probably contain some high-molecular-weight material with a relatively high E_{280} . Even when an effective DEAE-cellulose chromatography step is included in the isolation procedure some of this material appears in the product. Experiments on enzyme isolated without the use of a Sephadex G-200 column suggest a Z -average molecular weight for the material of 150000. This preparation contained about 10% impurity and so the molecular weight of the impurity is approximately 320000. This high-molecular-weight material at this concentration was not readily detectable by sedimentation-velocity measurements or by polyacrylamide-gel electrophoresis, and only became apparent during sedimentation-equilibrium studies or chromatography on Sephadex G-200. Thus future analytical work with rabbit muscle glycerol phosphate dehydrogenase should use only material that satisfies at least one of these criteria.

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References

- Ankel, H., Bücher, Th. & Czok, R. (1960) *Biochem. Z.* **332**, 315–327
- Beisenherz, G., Bücher, Th. & Garbade, K.-H. (1955) *Method Enzymol.* **1**, 391–397
- Brosemer, R. W. & Kuhn, R. W. (1969) *Biochemistry* **8**, 2095–2105
- Celliers, P. G., Stock, A. & Pfeleiderer, G. (1963) *Biophys. Acta* **77**, 577–591
- Clarke, J. T. (1964) *Ann. N.Y. Acad. Sci.* **121**, 428–436
- Cremona, T. & Singer, T. P. (1964) *J. Biol. Chem.* **239**, 1466–1473
- Dalziel, K. (1962) *Biochem. J.* **84**, 244–255
- Dalziel, K. (1963) *J. Biol. Chem.* **238**, 2850–2858
- Edelstein, S. J. & Schachman, H. K. (1967) *J. Biol. Chem.* **242**, 306–311

- Fisher, H. F., Adija, D. L. & Cross, D. G. (1969) *Biochemistry* **8**, 4424–4431
- Fondy, T. P., Ross, C. R. & Sollohub, S. J. (1969) *J. Biol. Chem.* **244**, 1631–1644
- Holbrook, J. J., Yates, D. W., Reynolds, S. J., Evans, R. W., Greenwood, C. & Gore, M. G. (1972) *Biochem. J.* **128**, 933–940
- Reithel, F. J. & Sakura, J. D. (1963) *J. Phys. Chem.* **67**, 2497–2498
- Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32–103
- Telegdi, M. (1968) *Biochim. Biophys. Acta* **159**, 227–235
- Telegdi, M. & Keleti, T. (1966) *Enzymologia* **31**, 83–91
- Telegdi, M. & Keleti, T. (1968) *Acta Biochim. Biophys. Acad. Sci. Hung.* **3**, 131–140
- Vallee, B. L., Hoch, F. L., Adelstein, S. J. & Wacker, W. E. C. (1956) *J. Amer. Chem. Soc.* **78**, 5879–5883
- Van Eys, J. (1960) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **19**, 26
- Van Eys, J., Nuenke, B. J. & Patterson, M. K. (1959) *J. Biol. Chem.* **234**, 2308–2313
- Van Holde, K. E. & Baldwin, R. L. (1958) *J. Phys. Chem.* **62**, 734–743
- Young, H. L. & Pace, N. (1958) *Arch. Biochem. Biophys.* **75**, 125–141
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317