# A NOVEL AFFINITY COLUMN FOR PURIFICATION OF GLYCEROL PHOSPHATE DEHYDROGENASE

James F. McGinnis and Jean de Vellis

Mental Retardation Research Center,
Neuropsychiatric Institute,
The University of California, Los Angeles, Ca. 90024

Received July 19,1974

SUMMARY: An affinity column was synthesized and utilized to partially purify glycerolphosphate dehydrogenase (L-glycerol-3-phosphate: $NAD^+$ oxidoreductase, E.C.1.1.1.8) from rat skeletal muscle. The novelty of the column resides in the fact that the ligand used, 6-phosphogluconic acid, is neither an inhibitor nor a substrate of the enzyme when free in solution but when immobilized on an agarose matrix, glycerol phosphate dehydrogenase binds to it with a high degree of specificity. The bound enzyme could be eluted by either increasing the ionic strength or by addition of its natural substrate, α-glycerol phosphate. Using a combination of these methods and ammonium sulfate precipitation GPDH was purified about 250 fold with a 75% yield within 24 hours.

INTRODUCTION: Glycerol phosphate dehydrogenase (GPDH, E.C.1.1.1.8) has been purified from a variety of tissues and in general the methods employed for its isolation involve a number of steps utilizing gel chromatography, ion exchange chromatography, ultracentrifugation and heat denaturation (1-6). Since the enzyme is relatively labile, in addition to the losses of activity encountered with each particular step in a purification procedure, there is also a decline in activity due to the total time required to perform all of the steps. For these reasons the yields obtained by various laboratories have been low (about 3 to 10%).

The clear and detailed description by Cuatrecasas (7) of the methodology used in the preparation of insoluble ligands has resulted in the extensive utilization of affinity chromatography for the purification of enzymes and other biologically active proteins. The specific enzymatic recognition of a substrate which has been covalently linked to an insoluble matrix results in the retention of the enzyme on the matrix. Proteins, with similar physical properties, but lacking a specific site for the ligand will not be retained by the column. The bound proteins can then be eluted either by varying factors which are known to affect biological reactions in general, such as the pH or ionic strength; or specific elution can be achieved by competing for the same sites with substrates or inhibitors of the biological reaction.

This report describes an affinity column which we designed and used to purify GPDH rapidly, and in high yield. The novelty of the

column resides in the fact that the ligand coupled to agarose, 6 phosphogluconic acid, is not a substrate, cofactor or inhibitor of GPDH activity when free in solution but when immobilized, GPDH binds to it. The bound GPDH can be eluted by substrate or by increasing the ionic strength of the buffer. Using a combination of these methods and an ammonium sulfate cut we rapidly purified GPDH from rat muscle by about 250 fold with a 75% yield.

MATERIALS AND METHODS: Ligand Coupling: Cyanogen bromide (CNBr) activation of agarose (Sepharose 4B, Pharmacia) and subsequent attachment of ligands were performed essentially as described by Cuatrecasas (7). Ligand coupling was monitored by reacting aliquots of each gel with 2,2, 6-trinitrobenzene sulfonate (Sigma Chemical Co.) (7). The spacer arm (1, 6-hexane diamine, ε-amino caproic acid or 3,3-diaminodipropylamine) to be coupled (4.3 mmoles/ml gel) was dissolved in a volume of glass distilled water equal to the volume of packed agarose and attached as described (7). Gels were washed extensively as described (8).

To 10 ml of the gel to which hexane diamine had been coupled, 10 ml of 0.1 M 6-phosphogluconic acid (6-PGA, Calbiochem) was added. The pH was adjusted to 6.0 with 1.0 N HCl and 4.0 ml (5 mmoles) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Biorad) were added dropwise with stirring at 20° C. The pH was repeatedly adjusted to 6.0 over a period of an hour and the suspension was then mechanically stirred for 20 hours at room temperature. This gel with a short spacer arm was then washed extensively as described (8). As a control ε-amino caproic acid was coupled to CNBr-activated agarose and phosphoethanolamine was subsequently attached to this using EDAC.

The agarose gel to which ε-amino caproic acid had been coupled was also used to synthesize the gel with the "long spacer arm". To 10 ml of this gel was added an equal volume of an aqueous solution containing 10 mmoles of diaminodipropylamine at pH 6.0. This slurry was stirred slowly and 5 ml of EDAC (1 mmole/ml) were added dropwise over a period of five minutes. The reaction was maintained at pH 6.0 for one hour and then allowed to proceed at room temperature for 20 hours. This gel was then washed extensively with $H_2O$, 6-PGA was attached and the gel subsequently washed in the same manner as was described for the short arm gel.

Muscle tissue, from the hind legs of Sprague-Dawley rats, was homogenized at 4° C in six volumes of 0.1 M Na-phosphate buffer, pH 7.5 containing 5 mM mercaptoethanol. Following centrifugation for one hour at 11,000 RPMs in a Sorval GSA head, ammonium sulfate was added to 40%,

stirred for one hour and the precipitate removed by centrifugation. The supernatant was adjusted to 80% ammonium sulfate, stirred one hour and again centrifuged. The precipitate was dissolved in 50 mM Tris-HCl, pH 8.3, 5 mM mercaptoethanol and any remaining ammonium sulfate was removed by passage of the sample through a Sephadex G-25 column equilibrated with the same buffer.

The optical density was monitored at 280 nm using an LKB UVICORD II which was connected to a log converter recorder resulting in direct recording of the optical density. Protein was determined according to the method of Lowry et al.(9) using 5 X crystallized bovine serum albumin (Sigma Chemical Co.) as a standard. GPDH was assayed as described previously (6).

RESULTS: The Fisher projection of sugars suggests that carbohydrates such as glucose (fig. 1) exist as open chains and that the terminal three

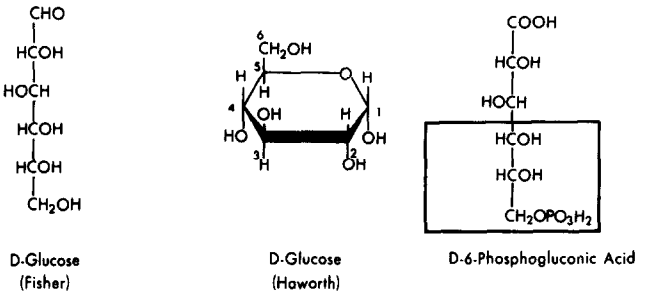


Fig. 1. Projections of glucose and 6-phosphogluconic acid.

carbons are similar to the three carbons of glycerol. However, the Fisher projection is more for convenience of writing and the true configuration of sugars is more accurately reflected by the Haworth projection (fig. 1) which shows glucose as a closed ring. A carbohydrate which does exist in an extended open chain form is gluconic acid and in 6-PGA, the terminal three carbons with their hydroxyls and phosphate group are structurally analogous to glycerol 3-Phosphate (fig. 1).

We first examined whether 6-PGA was a substrate or an inhibitor of GPDH using purified rabbit muscle GPDH (Calbiochem). When tested at concentrations up to 10 mM, 6-PGA did not act as a substrate nor did it inhibit the oxidation of α-GP. It also had no effect on the GPDH activity when assayed using dihydroxyacetcne phosphate as substrate.

This lack of recognition of 6-PGA in solution did not preclude an interaction with the immobilized ligand. Since GPDH has a relatively high Km (0.3 mM) for its natural substrate α-GP, 6-PGA was coupled to agarose by means of a hexane diamine spacer arm in order to increase the possibility of an interaction (10). The ability of this gel to bind GPDH was determined using purified rabbit muscle GPDH (fig. 2A). All of the en-

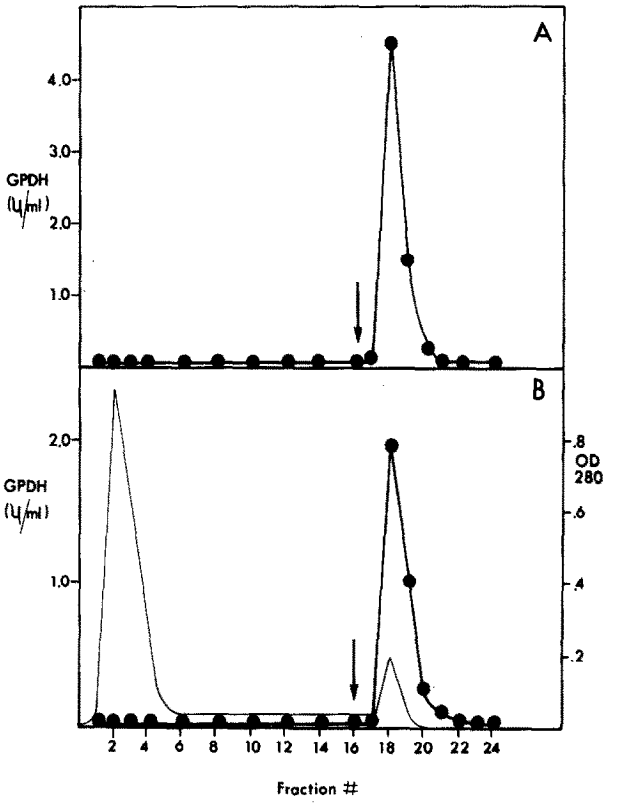


Fig. 2. Retention of GPDH on a 6-PGA "short arm" column and subsequent elution with 0.5 M NaCl (at arrow).
(A) Purified rabbit muscle GPDH.
(B) Rat muscle homogenate.

zyme applied was retained and none was detected in the eluant even after forty column volumes of buffer had passed through. The enzyme was eluted with 95-100% recovery by washing the column with 0.5 M NaCl in the same buffer.

We felt it was also necessary to demonstrate that the column would remove GPDH from a mixture of proteins. Therefore, a sample of rat muscle homogenate (see Methods) was applied to a small analytical column

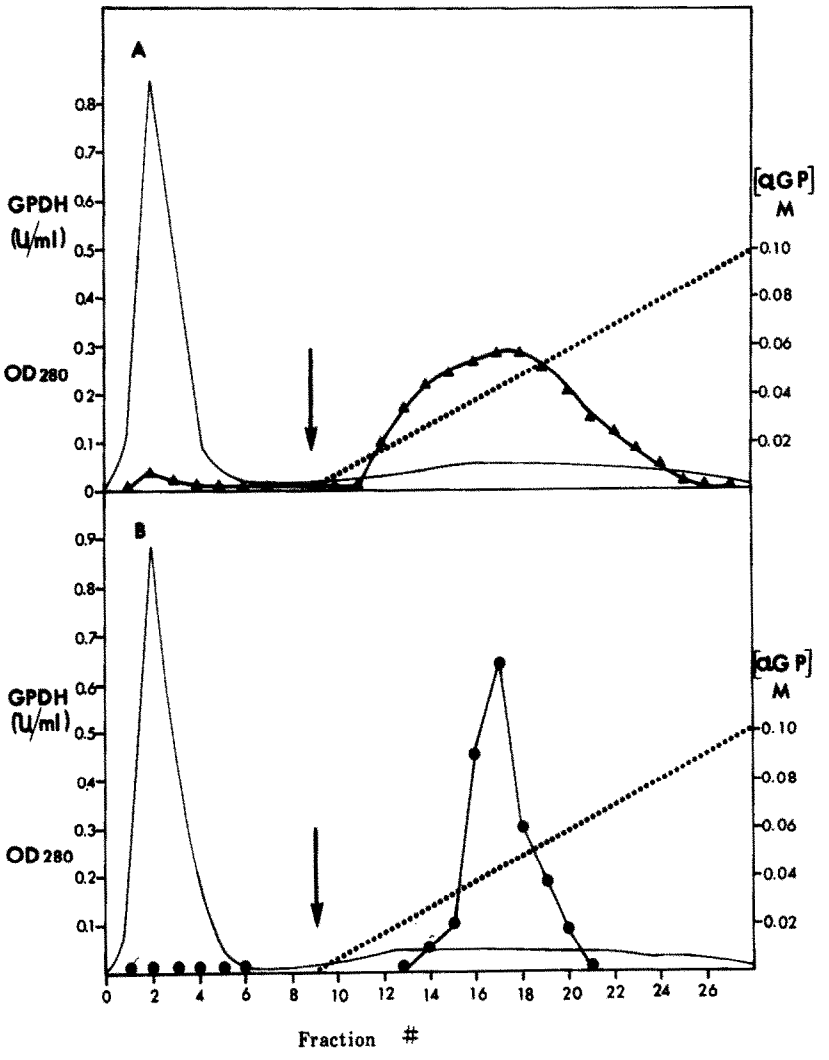


Fig. 3. Substrate elution of GPDH. Small columns (1.3 ml bed volume) were equilibrated with 50 mM tris. HCl, pH 8.3, 5 mM mercaptoethanol and a sample of rat muscle homogenate (0.4 ml) was applied to each. The columns were washed with buffer and a 60 ml gradient of αGP (0→ .1M) was attached at the point indicated by the arrow. The absorbance was monitored at 280 nM (———) and the GPDH assayed as described (6).
(A) Short spacer arm.
(B) Long spacer arm.

---

and the absorbance ($OD_{280}$) and enzyme activity in the effuent determined (fig. 2B). Although all of the GPDH was bound by the column, subsequent elution indicated that other proteins ($OD_{280}$) were also being retained Since the phosphate groups might be expected to remove proteins including

GPDH simply on the basis of its ionic charge, we examined the retention of GPDH on a control column containing a terminal phosphate on a similar spacer arm (agarose-ε-aminocaproic acid-phosphethanolamine). None of the GPDH was retained on the column indicating that the phosphate group extended out from the matrix on a spacer arm, was not sufficient to bind GPDH. In addition, GPDH does not bind to unsubstituted agarose.

Because other proteins were bound by the 6-PGA column, we attempted to differentially elute the enzyme using a substrate gradient (fig. 3A). This did separate most of the UV absorbing material from GPDH but under these conditions the enzyme was eluted as a very broad peak. To increase the resolution (1) we doubled the length of the spacer arm between the agarose and the matrix. Under the same conditions as were employed with the "short" spacer arm (fig. 3A), substrate elution from the matrix having a long arm (fig. 3B) resulted in a sharp profile of GPDH activity and a corresponding decrease in the amount of $OD_{280}$ absorbing material.

In order to optimize the purification, a number of parameters were varied and those which improved the yield and/or resolution were selected. GPDH will bind in the absence of added cofactor and the presence of NAD,

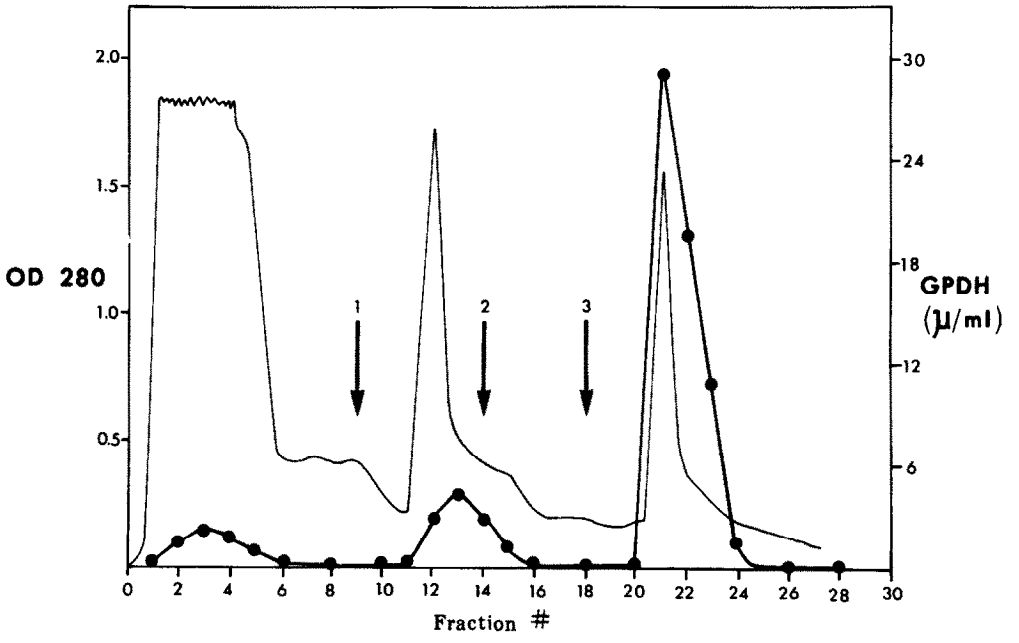


Fig. 4. Large column - Step wash. Rat muscle homogenate (12.0 ml, 28 mg protein/ml) was applied to a column 20 ml bed vol. At arrow #1, 20 ml of 0.1 M NaCl in 50 mM tris. HCl pH 8.3, 5 mM mercaptoethanol was applied. At the 2nd arrow, 20.0 ml of buffer alone was applied and finally (arrow #3) 0.1 M α-GP in the same buffer was applied. Absorbance (———) and GPDH activity (-●-) were determined as described.

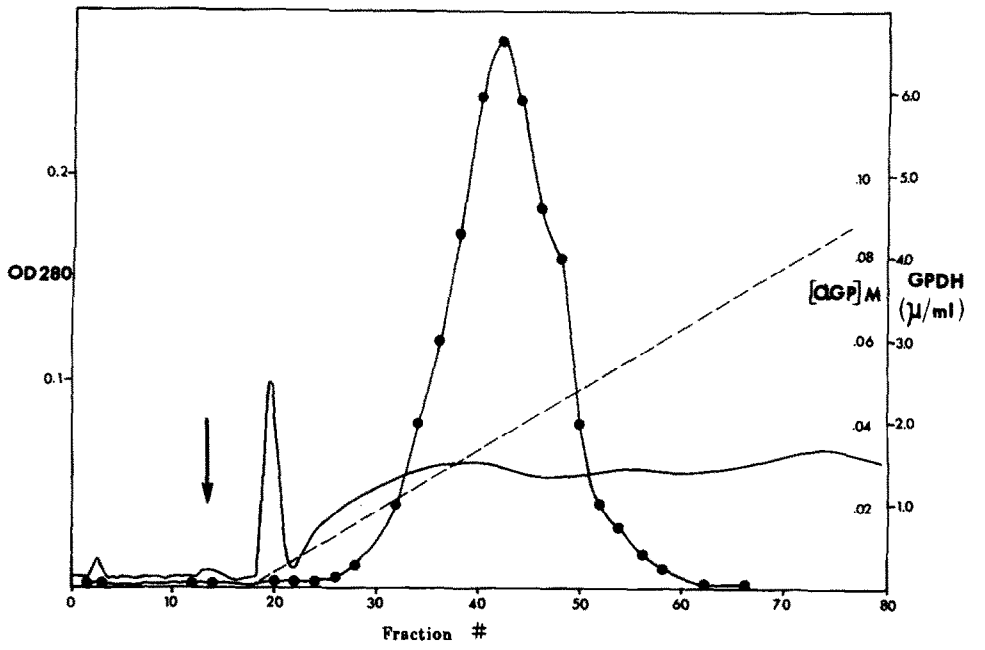


Fig. 5. Large column - gradient elution. The enzyme from the step wash (arrow #3) was precipitated, resuspended in buffer and applied to a column containing 6.0 ml of the same gel. After washing with buffer a 100 ml linear gradient of α-GP was started (arrow). Fractions were collected and assayed for enzyme activity as described.

---

$NADH_2$, ADP or AMP at contrations from 0.5 to 10 mM, did not affect the binding or elution of GPDH. Dihydroxyacetone phosphate (2.5 mM) in the presence or absence of $NADH_2$ did not elute the bound enzyme and neither did 10 mM 6-PGA.

Having determined optimum conditions for utilizing the affinity matrix, the purification of larger amounts of enzyme was attempted. The results of such an experiment are seen in fig. 4. Very little GPDH was in the flow-through peak which contained most of the $OD_{280}$ material and only a small amount was eluted with the salt wash. The enzyme activity in these fractions, when put onto a fresh column, was completely retained indicating that a different isozyme of GPDH was not involved. However, more than 80% was recovered following the step wash with substrate. The GPDH in this peak was concentrated, desalted and applied to a 6.0 ml column equilibrated with the same buffer and subsequently eluted with a substrate gradient from 0 to 0.1 M α-GP in 50 mM tris HCl pH 8.3 (fig. 5). The sharp $OD_{280}$ peak at the beginning of the gradient does not contain

GPDH and does not appear if the first column (fig. 4) is washed with three volumes of 0.1 M NaCl prior to elution with 0.1 M α-GP. It is also not seen if the first column is eluted directly with an α-GP gradient, but the yield and purification are not as high under these conditions.

The results of this purification method are summarized in Table 1.

Table 1

SUMMARY OF PURIFICATION

| Treatment | Total GPDH Units | % Yield | Total Protein(mg) | Specific Activity | Relative Specific Activity |
|---|---|---|---|---|---|
| 40 - 80% AS | 306 | 100 | 346 | 0.842 | 1 |
| Column 1 (step elution[a]) | 250 | 81.8 | 29.0 | 8.62 | 10 |
| Column 2 (step elution[b]) | 231 | 75.6 | 1.74 | 133.3 | 158 |

(a) Step elution was as described for Figure 4.

(b) Gradient elution was as described for Figure 5.

The final specific activity represents an increase of about 250 fold over the level found in the supernatant following centrifugation (specific activity = 0.550 U/mg P) and about 160 fold over the specific activity of GPDH in the ammonium sulfate precipitated material. The homogenization, ammonium sulfate precipitation, and first affinity column elution were performed in one day and the second column was eluted overnight and assayed on the following day. Thus in addition to the high yield obtained by this method, the time required to purify GPDH is decreased almost tenfold.

DISCUSSION: Having recognized the structural similarity between glycerol and the Fisher projection of sugars, and being aware of the extended chain form of gluconate, we coupled 6-PGA to agarose with the anticipation that such a column would specifically remove GPDH from solution.

The data presented indicate that this column does specifically retain GPDH, and that its subsequent elution results in the recovery of more than 75% of the enzyme in a purified form. Neither the retention of GPDH nor its elution was affected by NAD or NAD analogues. The specific activity of the purified enzyme (133 U/mg ) compares favorably with the specific activity of GPDH purified to homogeneity from rat brain (152 U/mg). Since the GPDH from these tissues has been shown to be immunologically (11,12) identical, the data suggest that at least 80% of the purified protein is actually GPDH.

NAD, AMP and other cofactor analogues have been coupled to insoluble matrices and used to purify NAD-dependent dehydrogenases (13-16). Recently a unique affinity gel was used to purify GPDH from a number of tissues (15). Using a hexamethylene diamine spacer arm, trinitrobenzene sulfonate was insolubilized on agarose and found to bind GPDH. Elution from this column and cofactor analogue columns was achieved either by increasing the ionic strength or by using NAD or NADH suggesting that the cofactor binding site on the enzyme was being competed for. It is possible that the sequential utilization of this type of column and one to which 6-PGA has been coupled can result in homogenous preparations of GPDH. In fact, a mixture of these materials might achieve this in a single step using as an eluant a solution containing both α-GP and NAD.

## REFERENCES


1. Fondy, T.P., Lesin, L., Sollohub, S.J. and Ross, C.R. J. Biol. Chem. 243, 3148-3160 (1968).
2. White, H.B., III and Kaplan, N.O. J. Biol. Chem. 244, 6031-6039 (1969).
3. Fondy, T.P., Ross, C.R. and Sollohub, S.J. J. Biol. Chem. 244, 1631-1644 (1969).
4. Fondy, T.P., Herwig, K.J., Sollohub, S.J. and Rutherford, D.B. Arch. Biochem. Biophys. 145, 583-590 (1971).
5. Otto, J., Raggi, A., Machleidt, W. and Bücher, T. Hoppe-Seyler's Z. Physiol. Chem. 353, 332-344 (1972).
6. McGinnis, J.F. and de Vellis, J. Biochim. Biophys. Acta. In press.
7. Cuatrecasas, P. J. Biol. Chem. 245, 3059-3065 (1970).
8. Cuatrecasas, P. and Parikh, I. Biochemistry 11, 2291-2299 (1972).
9. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. J. Biol. Chem. 193, 265-275 (1951).
10. Cuatrecasas, P. Advances in Enzymology 36, 29 (1972).
11. McGinnis, J.F. and de Vellis, J. Fed. Proc. 32, 604 (1972).
12. Lee, Y.P. and Choy, P.C.C. J. Biol. Chem. 249, 476-481 (1974).
3. Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. Biochem. J. 127, 625-631 (1972).

14. Lowe, C.R., Mosbach, K. and Dean, P.D.G. Biochem. Biophys. Res. Commun. 48, 1004-1010 (1972).
15. Lowe, C.R., Harvey, M.J., Craven, D.B., Kerfoot, M.A., Hollows, M.E. and Dean, P.D.G. Biochem. J. 133, 507-513 (1973).
16. Barry, S. and O'Carra, P. Biochem. J. 135, 595-607 (1973).
17. Kornbluth, R., Ostro, M.J., Rittmann, L.S. and Fondy, T.P. FEBS Letters 39, 190-194 (1974).