of the enzyme-substrate complex, as well as the thermodynamic constants of its activation are very close to each other, under all conditions.

A study of the effect of surface-active substances other than Tween 80 has shown that groups of non-ionic and cationic surface-active substances are capable of augmenting the enzymic activity of carboxypeptidase, and that only the group of anionic surface-active substances is without this property.

## BIBLIOGRAPHIE


[1] R. LUMRY, E. L SMITH ET R. GLANTZ, *J. Am. Chem. Soc.*, 73 (1951) 4330.
[2] L. GORINI ET J. LABOUESSE-MERCOUROFF, *Biochim. Biophys. Acta*, 13 (1954) 291.
[3] R. LUMRY ET E. L. SMITH, *Discussions Faraday Soc.*, 20 (1955) 105.
[4] S. YANARI ET M. A. MITZ, *J. Am. Chem. Soc.*, 79 (1957) 1154.
[5] J. LABOUESSE, *Biochim. Biophys. Acta*, 26 (1957) 52.
[6] E. L. SMITH ET A. STOCKELL, *J. Biol. Chem.*, 207 (1954) 501.
[7] N. M. GREEN ET H. NEURATH, dans H. NEURATH ET K. BAILEY, *The Proteins*, Vol. II, part B, Academic Press, Inc., New-York, 1954, p. 1057.
[8] E. FISCHER ET W. SCHOELLER, *Ann.*, 357 (1907) 1.
[9] K. HOFMANN ET M. BERGMANN, *J. Biol. Chem.*, 134 (1940) 225.
[10] E. FISCHER ET O. WARBURG, *Ann.*, 340 (1905) 123.
[11] M. A. STAHMANN, J. S. FRUTON ET M. BERGMANN, *J. Biol. Chem.*, 164 (1946) 753.
[12] T. CURTIUS ET E. MULLER, *J. prakt. Chem.*, (2), 70 (1904) 223.
[13] S. MOORE ET W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
[14] G. S. EADIE, *J. Biol. Chem.*, 146 (1942) 85.
[15] H. NEURATH ET G. W. SCHWERT, *Chem. Revs.*, 46 (1950) 69.
[16] A. E. STEARN, *Advances in Enzymol.*, 9 (1949) 25.
[17] H. LINDLEY, *Advances in Enzymol.*, 15 (1954) 271.
[18] K. J. LAIDLER, *Discussions Faraday Soc.*, 20 (1955) 83.
[19] J. E. SNOKE ET H. NEURATH, *J. Biol. Chem.*, 181 (1949) 789.


Reçu le 2 Septembre 1957

# ADENOSINE MONO-, DI- AND TRIPHOSPHATE, PYRUVIC KINASE, HEXOKINASE AND POLYNUCLEOTIDE PHOSPHORYLASE ASSAY*

SANTIAGO GRISOLIA**, LEWIS C. MOKRASCH AND VERNE D. HOSPELHORN

*McIlvain Laboratories and Lerrigo Laboratories, Department of Mecidine, University of Kansas Medical Center, Kansas City, Kansas (U.S.A.)*

The procedures described here provide fast, simple, reproducible and reasonably specific assays for adenylic acid, AMP***, ADP and ATP as well as for the assay of pyruvic kinase, hexokinase and polynucleotide phosphorylase. All procedures are based on the interdependence of the components of reactions:

* Supported by Grants No. 67 The Helen Hay Whitney Foundation, H 1925 National Institutes of Health, Life Insurance Medical Research Fund and the Kansas Heart Association.

** Established Investigator of the American Heart Association.

*** The following abbreviations are used in this paper: AMP, ADP, ATP, 5′-adenosine mono-, di-, or triphosphate respectively; PGA 3-D-phosphoglyceric acid; 2-PGA, 2-D-phosphoglyceric acid; PEP, phosphoenol pyruvic acid; mutase, 3-phosphoglyceric acid mutase; 2,3-DPGA, 2,3-diphosphoglyceric acid. RNA, ribose nucleic acid; Tris, tris (hydroxymethyl)-aminomethane; $AMPCO_2$, the active intermediate described by BACHHAWAT *et al.*[16].

*References p. 354.*

1 3-PGA ⟷ 2-PGA
2 2-PGA ⟷ PEP
3 PEP + ADP ⟶ Pyruvate + ATP
4 ATP + glucose ⟶ glucose-6-phosphate + ADP
5 2'ADP ⟷ AMP + ATP

Reaction 1, catalyzed by phosphoglyceric acid mutase from yeast or muscle, is dependent on 2,3-DPGA[1,2,3] and this fact was used previously in this laboratory to develop a micromethod for 2,3-DPGA[4]. The methods described here use the same principles.

## METHODS AND MATERIALS

3-PGA containing 0.26% DPGA[4] was obtained from Schwartz laboratories. AMP, ADP and ATP were Sigma or Pabst products. Hexokinase (fraction 3) was prepared as described by BERGER *et al.*[5]. It can be lyophilized or kept frozen in glucose with little loss of activity. Both methods of preservation have been used in this laboratory for over 3 years with excellent results. Some 10 preparations of hexokinase obtained in this laboratory* have invariably yielded preparations rich in 3-phosphoglyceric mutase** and enolase but low in pyruvic kinase. The rabbit-muscle acetone powder, obtained as described previously[3], retains its activity very well for over a 2-year period when stored dry and in the cold. The mutase-enolase was prepared by mixing crystalline mutase[2] and purified enolase[4]. Pyruvic kinase was obtained as described by KORNBERG AND PRICER[7]. The product of approximately 50% purity before gel absorption was used and kept lyophilized***. Pyruvic acid was determined by the method of FRIEDEMANN AND HAUGEN[8] as modified by KACHMAR AND BOYER[9] and inorganic phosphate by that of GOMORI[10]. Protein was measured by a modified biuret method[3].

All incubations were carried out, unless specified otherwise, in a 2-ml final volume and at 38° for 10 min. The incubation mixtures were deproteinized by the addition of 2 ml 0.5 *M* $HClO_4$ or by heating followed by centrifugation. In all cases pyruvate estimation was used as an index of the extent of the reaction. Also in all cases, a blank for the components of the reaction mixtures was included.

### *Assay for ADP and ATP*

The conditions for the method are as follows: to a nucleotide sample (0.005–0.1 μmole) in 1 ml, add 1 ml of a mixture containing potassium 3-PGA, 50 μmoles; Tris buffer pH 7.4, 150 μmoles; glucose, 100 μmoles; $MgSO_4$, 20 μmoles; lyophilized hexokinase, 0.7 mg protein; pyruvic kinase, 0.25 mg protein. Under these conditions the ratio of pyruvate formation to ADP or ATP is about 75 to 1. Linearity is seen up to about 0.2 μmoles of ADP or ATP. Since the method is sensitive to salt concentration, it is advisable, in spite of the high salt concentration used purposely in the procedure, to carry standards through any manipulation used with samples for analysis. The convenience of this is further emphasized by inspection of Fig. 1.

### *Assay for AMP*

The components and conditions for AMP assay are as described for ADP and ATP, except that the incubation is carried out for 20 to 30 min, the final volume decreased to 1 ml and the pyruvic kinase is replaced by a water extract of rabbit muscle acetone powder (about 0.5 mg protein of the 2000 × 9 supernatant fluid).

* We wish to thank Drs. D. P. WALLACH, V. W. RODWELL and S. CARDOSO for preparing several batches of hexokinase, and Dr. J. C. TOWNE for help with some of the methods here described.

** This and other observations led to the utilization of baker's yeast for the purification and crystallization of 3-PGA mutase[2].

*** We have had no success in several attempts to repeat the last step of the KORNBERG AND PRICER procedure for pyruvic kinase[7].

*References p. 354.*

Because there is essentially no reaction until optimum amounts are reached, it is best to determine the amount required for any particular acetone powder. For example, with our preparations we obtained linear response to AMP concentrations with the amount of protein indicated. When half of this quantity was used, the response was not linear and pyruvate liberation decreased by about 90%. As discussed below, this may indicate the need of small amounts of ADP or ATP and myokinase to prime the reaction.

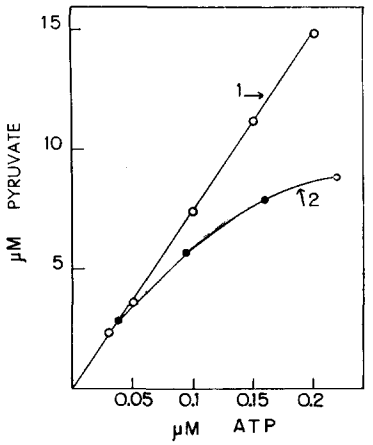


Fig. 1. Standard curves for ATP estimation. The conditions of assay were as stated in the text. Curve No. 1, ATP standard. Curve No. 2, recovery curve at increasing salt concentration. 1 μmole ATP in 1 ml was mixed with 1 ml 5% $HClO_4$, 2.2 ml 0.33 *N* KOH were added, the tubes were chilled in ice for 5′, centrifuged in the cold and aliquots of the neutralized supernatants were tested as stated in the text. The calculated ATP concentrations are plotted in the ordinate *versus* the pyruvate found in the assay.

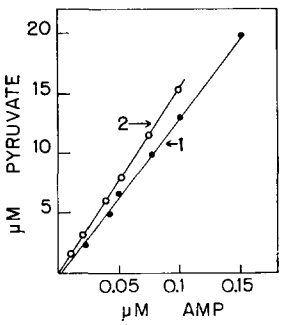


Fig. 2. Standard curve for AMP. The conditions of assay were as stated in the text. Curve 1, 15 min incubation, rabbit-muscle acetone-powder extract 7 days old (frozen at —20°). Curve No. 2, rabbit-muscle acetone-powder extract 14 days old (frozen at —20°) 30 min incubation.

The muscle extract is fairly stable for 1–2 weeks at —20°, however the slope of the curve will vary with the storage time and therefore it is again essential to carry standards from day to day. The method is quite sensitive since the ratio of AMP to pyruvate production under the prescribed conditions is about 1 to 150. This is further illustrated in Fig. 2.

*Pyruvic kinase and hexokinase assay*

The conditions for assay are as follows: enzyme samples, 0.5 ml. Add 0.7 mg protein of lyophilized hexokinase in 0.5 ml when assaying for pyruvic kinase, or a mixture of 0.5 mg of pyruvic kinase, 1000 units of mutase[2] and 100 units of enolase (2,4) in 0.5 ml if assaying for hexokinase. Add 1 ml of a mixture containing the following components in μmoles: potassium 3-PGA, 50; Tris buffer pH 7.2, 100; $MgSO_4$, 20; glucose, 100; and 1 or 2 μmoles of ATP when assaying for hexokinase or pyruvic kinase respectively.

The method for pyruvic kinase is similar to the one described by KACHMAR AND BOYER[9], however the inexpensive 3-PGA, mutase and enolase (present in the hexokinase) are used instead of PEP.

*References p. 354.*

As an example, with hexokinase at stage 3[5], a 20-fold linear range of enzyme concentration was measured. With pyruvic kinase the range is somewhat more limited due to the presence of traces of pyruvic kinase and other contaminating enzymes in the hexokinase preparation*.

*Polynucleotide phosphorylase assay*

The preparation of the chicken-liver fractions containing the phosphorylase used has been described[11].

The incorporation of labeled 5'-uridylic acid into RNA in the presence of ATP and nucleotide phosphokinases and the reverse reaction, namely phosphorolysis of added RNA**, measures the enzyme activity.

The conditions for the assay are as follows: 0.1 to 0.4 ml enzyme; 30 μmoles Tris-maleate buffer pH 7.6 to 8.0; 5 mg purified yeast RNA; 3 μmoles $MgSO_4$; 50 μmoles phosphate buffer pH 7.6 to 8.0, and distilled water to 1.0 ml; the control tube is obtained by the omission of phosphate. Incubation time 30 min. The reaction is stopped by immersion of the tubes in boiling water for 2 min. After centrifugation, 0.1 ml of the supernatant fluid is taken for analysis as described in the section for AMP.

In the cruder preparations the assay for phosphorylase may give low values for the activity owing to the presence of enzymes deaminating the adenine nucleotides. To reduce the blank values in the assays, the phosphorylase preparations may be dialysed for 4–5 h at 0° against the Tris-maleate buffer used for the assay.

## DISCUSSION

The methods described here are all based on the colorimetric estimation of pyruvate. They are very sensitive and rapid and require simple equipment. Some of them have been in use in this laboratory for over three years and have proved to be satisfactory to a number of co-workers who have used them. In the case of nucleotide determinations, the catalytic dependence of the enzymes is so marked that about a 150 to 1 ratio of pyruvate to nucleotide is obtained, and therefore endogenous pyruvate in samples is of little consequence and in most cases it is not necessary to correct for it. The method is however, very sensitive to salt and pH and when measuring unknowns it is best to carry standards through all manipulations. It must be mentioned here that the estimation of AMP appears to be dependent on the presence of small amounts of ATP or ADP and of myokinase[13] in the muscle preparation. The presence of the enzyme postulated by MEYERHOF AND OSPER[14] catalyzing the interaction of AMP

* The hexokinase assay may be misleading with crude preparations having low activity, high "ATP-ase" activity together with high 1,3-diphosphoglycerate kinase activity will obscure the hexokinase activity measurements. Phosphate as well as pyruvate estimations will reveal this difficulty. This has proved to be the case in experiments with lung homogenates (V.H. unpublished). It should be emphasized that the finding of a selective inhibitor for the 1,3-diphosphoglycerate kinase would make this procedure more specific. It would permit also the measurement of ATP-ase activity without interference or inhibition of the enzyme(s) by ADP.

** It has been noted that the partially purified enzyme used will attack the RNA of yeast, pigeon liver and chicken liver. This lack of species specificity is in accord with the findings of GRUNBERG-MANAGO, ORTIZ AND OCHOA[12] who found that their *Azotobacter* enzyme would attack a variety of RNA's.

*References p. 354.*

and PEP has not been excluded, however*. Although no extensive study of nucleotide specificity has been conducted, 3'-adenylic acid, IMP, and 3'- and 5'-uridylic acids cannot replace the adenine nucleotides.

The proposed methods are easily applied to optically opaque solutions and because of the ease of handling, the accurate temperature control and the large number of samples that can be assayed at the same time seem to offer definite advantages over other excellent methods used at present, such as the manometric method for estimation of hexokinase[16] or the optical method of OCHOA[17] for nucleotide phosphorylase. The first is time-consuming and the second can be used only with preparations of high purity[17]. When convenient, estimation of inorganic phosphate[10], or the use of proper blanks, provides additional assurance that other reactions are not interfering with the estimations based on the methods described here.

## SUMMARY

Fast, simple, reproducible and reasonably specific assays for 0.03 $\mu$mole or less, per ml for adenylic acid, adenosine di- and triphosphates, and for pyruvic kinase, hexokinase and polynucleotide phosphorylase are presented. Some of the limitations of the methods described are discussed.

## REFERENCES

1 E. W. SUTHERLAND, T. POSTERNAK, and C. F. CORI, *J. Biol. Chem.* 181 (1949) 153.
2 V. W. RODWELL, J. C. TOWNE AND S. GRISOLIA, *Biochim. Biophys. Acta*, 20 (1956) 394.
3 V. W. RODWELL, J. C. TOWNE AND S. GRISOLIA, *J. Biol. Chem.*, 228 (1957) 875.
4 J. C. TOWNE, V. W. RODWELL AND S. GRISOLIA, *J. Biol. Chem.*, 226 (1957) 777.
5 L. BERGER, M. W. STEIN, S. P. COLOWICK AND C. F. CORI, *J. Gen. Physiol.*, 29 (1946) 379.
6 S. RATNER, in COLOWICK AND KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press. Inc., New York, 1955, 356.
7 A. KORNBERG AND W. E. PRICER, *J. Biol. Chem.*, 193 (1951) 481.
8 T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
9 J. F. KACHMAR AND P. D. BOYER, *J. Biol. Chem.*, 200 (1953) 669.
10 G. GOMORI, *J. Lab. Clin. Med.*, 27 (1941–'42) 955.
11 L. C. MOKRASCH AND S. GRISOLIA, *Biochim. Biophys. Acta*, 27 (1958) 226.
12 M. GRUNBERG-MANAGO, P. ORTIZ AND S. OCHOA, *Science*, 122, (1955) 907.
13 S. P. COLOWICK, AND H. M. KALCKAR, *J. Biol. Chem.*, 148 (1943) 117.
14 O. MEYERHOF AND P. OSPER, *J. Biol. Chem.*, 179 (1949) 1371.
15 S. P. COLOWICK AND H. M. KALCKAR, *J. Biol. Chem.*, 137 (1941) 789.
16 B. K. BACHHAWAT, J. F. WOESSNER AND M. J. COON, *Federation Proc.*, 15 (1956) 214.
17 S. OCHOA, in W. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, Johns Hopkins Press, Baltimore, 1956, p. 615.

Received December 18th, 1957

* Unpublished experiments of S. GRISOLIA, J. C. TOWNE, B. K. BACHHAWAT AND M. J. COON showed that synthetic $AMPCO_2$ but not its ester [16] reacted with PEP in the presence of a lyophilized ammonium sulfate fraction of rabbit muscle[6] and $Mg^{++}$ to give pyruvate and ADP. Whether or not the intermediate formation of oxalacetic acid occurs is not known. AMP could not replace $AMPCO_2$ in this system. In the presence of versene there was no reaction. Thus it is not possible to decide for the moment whether myokinase (and traces of ADP and/or ATP) or some other reaction is responsible for the effect observed.