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Spectrophotometric Determination of Fructose-1:6-Diphosphate, Hexosemonophosphates, Adenosinetriphosphate and Adenosinediphosphate

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(*Received* 4 *June* 1952)

Spectrophotometric methods are now widely used for following the course of certain enzymic reactions. When the reverse reaction is slight, it is possible to adapt the procedure so that it becomes a specific and usually very sensitive method of measuring the concentration of the reactants. Reactions involving oxidized or reduced diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) have been particularly useful, since the reduced coenzyme absorbs strongly in the near ultraviolet (Warburg & Christian, 1936) and a number of enzymes catalysing the reaction between various intermediary metabolites and the coenzyme can be prepared. Examples are the estimation of pyruvate and malate (Ochoa, Mehler & Kornberg, 1948), *iso*citrate (Ochoa, 1948), α-ketoglutarate (Kornberg & Pricer, 1951*a*) and glucose-6-phosphate (Ochoa, Salles & Ortiz, 1950; Slein, 1950; Kornberg & Pricer, 1951*b*).

In connexion with investigations of oxidative phosphorylation, a very sensitive method of determining fructose-1:6-diphosphate (HDP) was required. Dr Racker suggested to me that his method (Racker, 1947) of measuring phosphohexokinase activity might be adapted for this purpose. The present paper describes the successful adaptation of this method for the estimation not only of HDP, but also of the hexosemonophosphates (glucose-6-phosphate, fructose-6-phosphate and glucose-1-phosphate) and adenosinetriphosphate (ATP) and adenosinediphosphate (ADP). It can also be adapted for the measurement of creatinephosphate. A preliminary account of this work has already been published (Slater, 1951).

PRINCIPLE OF METHODS

Procedure A. In the presence of rabbit-muscle fraction *A* (see Methods), HDP reacts with an excess of reduced DPN, according to the following scheme:

(1) HDP → glyceraldehydephosphate + dihydroxyacetonephosphate (*aldolase*),

(2) Glyceraldehydephosphate → dihydroxyacetonephosphate (*triosephosphate isomerase*),

(3) 2 Dihydroxyacetonephosphate + 2 (reduced DPN) → 2 glycerolphosphate + 2DPN (*glycerolphosphate dehydrogenase*).

Overall reaction (*A*): HDP + 2 (reduced DPN) → 2 glycerolphosphate + 2 DPN.

The rabbit-muscle fraction *A* contains the necessary enzymes shown in brackets after the equations. Since the equilibrium constant of reaction (3) is $1{\cdot}4 \times 10^4$ at 22° and pH 7 (Baranowski, 1949), the overall reaction *A* proceeds virtually to completion in the direction shown and the disappearance of reduced DPN, determined by the decrease of optical density at 340 mμ., is a measure of the HDP concentration. The alternative reaction of glyceraldehydephosphate, namely oxidation by DPN in the presence of inorganic phosphate and glyceraldehydephosphate dehydrogenase, does not occur with the rabbit-muscle fractions used (see below).

In this procedure, and throughout the paper, it should be understood that any glyceraldehydephosphate or dihydroxyacetonephosphate present will be included in the estimation of HDP. For most purposes, the estimation of the sum of HDP and the two triosephosphates is more useful than the estimation of only the HDP. L-Sorbose-1-phosphate, which liberates one molecule of dihydroxyacetonephosphate when treated with aldolase (Lardy, Wiebelhaus & Mann, 1950) would no doubt behave in the same way as triosephosphate.

Procedure B. If Mg^{++}, excess ATP and rabbit-muscle fraction *B* (see Methods) are added to the components used in procedure *A*, the three hexosemonophosphates which appear in glycolysis will react as follows:

(4) Glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate (*phosphoglucomutase* and *hexosemonophosphate isomerase*),

(5) Fructose-6-phosphate + ATP $\rightarrow$ HDP + ADP (*phosphohexokinase*),

(6) ADP $\rightarrow \frac{1}{2}$ ATP + $\frac{1}{2}$ adenylic acid (*myokinase*),

(*A*) HDP + 2 (reduced DPN) $\rightarrow$ 2 glycerolphosphate + 2 DPN.

Overall reaction (*B*): HMP + $\sim$P + 2 (reduced DPN) $\rightarrow$ 2 glycerolphosphate + 2 DPN,

where HMP is the sum of the three hexosemonophosphates and $\sim$P represents the reactive energy-rich phosphate groups of ATP and ADP. All the necessary enzymes are present in rabbit-muscle fraction *A* or *B*.

This reaction can be used in two ways: (*a*) in the presence of excess $\sim$P the disappearance of reduced DPN is a measure of the total concentration of the three hexosemonophosphates + HDP; (*b*) in the presence of excess HMP the disappearance of reduced DPN is a measure of the HDP + total concentration of $\sim$P of ATP and ADP.

Procedure C. A separate sample of the solution to be analysed is treated with glucose, Mg^{++} and yeast hexokinase. When reaction (*C*)

(*C*) ATP + glucose $\rightarrow$ glucose-6-phosphate + ADP (*hexokinase*)

has reached completion, the hexokinase is vated by the addition of trichloroacetic ac removed by centrifugation and HMP estima the neutralized supernatant by procedure *B*.

EXPERIMENTAL

Materials

Rabbit-muscle fraction A. This was the prepara scribed by Racker (1947) as 'glycerophosphate d genase and aldolase'. Racker's procedure was f exactly. The $(NH_4)_2SO_4$ paste was stored at −15 solution freshly prepared for each day's analyses. Tl was dissolved in ice-cold water and filtered to give a tration of about 20 mg. protein/ml. The solution was ice. The actual concentration used depended u activity of the preparation, which declined on sto retained sufficient activity for about 3 months), an presence of inhibiting substances, such as trichloro in the solutions to be analysed. The concentration of used in the test should be such that the reaction is c within 10 min., preferably 5 min.

Rabbit-muscle fraction B. This was essentially F (1947) phosphohexokinase preparation. The pre obtained between 0·2 and 0·5 saturation with (N during the preparation of rabbit-muscle fraction dissolved in about 50 ml. 0·01 M-phosphate buffer, and dialysed overnight against 0·35 saturated (NH pH 7·6. The precipitate was removed by centrifug the cold and the supernatant brought to 0·5 saturati 0·3 vol. saturated $(NH_4)_2SO_4$, pH 7·6. The precipit collected by centrifugation in the cold and suspend little 0·01 M-phosphate buffer, pH 7·6. The paste, wh stored at −15°, retained sufficient activity for 3 months. A fresh solution was prepared for eac analyses by diluting tenfold with ice-cold 0·025 M glycine buffer, pH 7·6. This solution is faintly clou does not need filtering.

Hexokinase. Two preparations of yeast hexokina used in this study: (*a*) purified hexokinase prepa fractionation with ethanol and adsorption on alum according to Berger, Slein, Colowick & Cori (1946 crude preparation prepared as follows.

Baker's yeast (6·4 kg.) was autolysed according procedure of Allfrey & King (1950). The only modific this procedure was to make the suspension 1 % with to glucose immediately after autolysis (cf. Berger *et al* The lower aqueous layer was filtered through a pad o Cel on a Büchner funnel. The filtrate was brought saturation by the slow addition of 350 g. $(NH_4)_2SO_4$/l. mixture filtered through large fluted papers overnig filtrate was brought to 0·75 saturation by the slow a of 138 g. $(NH_4)_2SO_4$/l. and the mixture filtered ove The precipitate was dissolved in water containin glucose, brought to pH 7·0 and diluted to 1 l. The s was brought to 0·55 saturation by the slow addi $(NH_4)_2SO_4$, allowance being made for the $(NH_4)_2SO$ precipitate. The pH was kept at 7·0 by the occ addition of N-KOH. The precipitate was removed b tion overnight, the filtrate brought to 0·75 saturation slow addition of 138 g. $(NH_4)_2SO_4$/l. and the pre collected by centrifugation. It was transferred with tion containing 0·01 M-acetate buffer (pH 5·4)—1 %

to a dialysis sac and dialysed against this solution until free from sulphate. The dialysed solution was stored at -15°.

A small yield of hexokinase of higher specific activity can be obtained by refractionation of this solution between 0·85 and 0·95 saturation with $(NH_4)_2SO_4$ (Holton, 1952).

Creatinephosphokinase. A preparation from rabbit muscle was kindly supplied by Dr B. Askonas.

Lactic dehydrogenase. This was purified by fractionation of rabbit-muscle fraction *A* with $(NH_4)_2SO_4$, according to the method of Korkes, Del Campillo, Gunsalus & Ochoa (1951). Lactic dehydrogenase crystallized at 0·55 saturation with $(NH_4)_2SO_3$ after standing for several days at 0°. The crystalline precipitate was collected by centrifugation, suspended in a little 0·01 M-phosphate, pH 7·6, and stored at -15°.

DPN. Several preparations of DPN ranging from 33 to 79% purity have been used in this study. Laboratory-prepared samples from yeast by an unpublished method of Ochoa, from liver by the method of LePage & Mueller (1949), and commercially available DPN are all suitable for the estimation of HDP, HMP or ATP. Especially purified DPN is, however, necessary for the estimation of $\sim$P, since many impure preparations contain considerable amounts of $\sim$P. A preparation 79% pure obtained by LePage & Mueller's method was particularly suitable. Dr R. K. Morton (private communication) has found that treatment of DPN, prepared by Ochoa's method, for 4 min. at pH 1·5 and 100° reduces the $\sim$P content to a level suitable for estimations of creatinephosphate.

Reduced DPN. This was prepared either by reduction with $Na_2S_2O_3$ according to Ohlmeyer's (1938) procedure or by reduction with alcohol and alcohol dehydrogenase (Racker, 1950; Bonnichsen, 1950). The following procedure has been found satisfactory.

A sample containing 15 mg. DPN is dissolved in 20 ml. water, 3 ml. 10 M-ethanol (aldehyde-free) added and the solution brought to pH 9·0 with N-KOH and 0·1 N-KOH. Approx. 1 mg. of crystalline yeast alcohol dehydrogenase (Racker, 1950) is added and the pH again adjusted. The solution is diluted to 30 ml. and the optical density at 340 mμ. followed in a 0·5 cm. cell keeping the pH at 9·0. When the optical density has reached a maximum (about 10 min.), the solution is immersed in a boiling-water bath for 5 min., cooled and filtered. The solution can be stored for approx. 2 weeks at -15°. Pure liver alcohol dehydrogenase (kindly supplied by Dr R. K. Bonnichsen) was also used satisfactorily. Since this enzyme has a lower turnover number than that from yeast, a somewhat longer time is required to obtain the maximum reading.

If the DPN sample is contaminated with heavy metals, a little 'Versene' (ethylenediamine tetraacetic acid) should be added before the alcohol dehydrogenase.

ATP. The barium salt of ATP was either obtained commercially or prepared in the laboratory from rabbit muscle by the procedure described by LePage (1949*a*), omitting the magnesium anaesthesia. The mercury precipitation was repeated as described therein. The barium salt was converted into a neutral solution of the potassium salt by passage through an ion-exchange column (Polis & Meyerhof, 1947; Rowles & Stocken, 1950). Following the advice of Dr S. M. Partridge, Dowex 50 was the resin used. This resin effected some purification of the commercial ATP by removing some of the adenylic acid. The procedure adopted was to treat the column with 2 N-HCl and then wash through with water until the eluate no longer turned Congo red paper blue. The barium ATP was dissolved in the minimum volume of cold N-HCl and, after filtration if necessary, poured through the column. The rate of flow (area approx. 1 sq.cm.) was 15 ml./hr. The ATP solution was followed by water. Collection of the eluate commenced when a drop turned Congo red paper blue and ceased when it no longer turned the colour. Alternatively, the optical density at 260 mμ. of the eluate was followed. The eluate was kept cold and finally neutralized with N-KOH. This solution is stable for several months at -15° (cf. Bailey, 1949).

ADP. This was prepared from ATP by the addition of glucose and hexokinase, following a procedure essentially the same as that used by Colowick & Kalckar (1943). The barium salt was isolated and converted into the potassium salt in the same way as for ATP.

Creatinephosphate. A sample of the crystalline sodium salt, prepared by the method of Ennor & Stocken (1948), was kindly supplied by Dr A. Narayanaswami.

Fructose-6-phosphate. A solution of the potassium salt was prepared from the commercial barium salt by means of the ion-exchange column procedure, as described for ATP. This solution contains very little inorganic phosphate.

Glucose-6-phosphate. A sample of the barium salt, prepared synthetically, was kindly supplied by Dr E. Racker. The solution of the potassium salt was prepared by treatment of the barium salt with K_2SO_4. There was no measurable inorganic phosphate in this solution.

Hexosemonophosphate. The supernatant obtained after precipitation of the barium salt of ADP from the reaction mixture after treatment of ATP with hexokinase and glucose (see above) was brought to pH 8·2 and treated with 4 vol. 95% (v/v) ethanol. After cooling in ice, the precipitate was collected by centrifugation and washed with 95% ethanol, followed by ether. The solution of the potassium salt was prepared by treatment of the barium salt with K_2SO_4. The molar concentrations of inorganic phosphate and ADP in this solution were respectively 0·07 and 0·24% that of the hexosemonophosphate. Since the hexokinase used in this preparation very likely contained hexosemonophosphate isomerase, this preparation is probably a mixture of glucose-6-phosphate and fructose-6-phosphate.

Glucose-1-phosphate. A sample of the dipotassium salt (dihydrate) was kindly supplied by Dr D. M. Needham.

Phosphoglyceric acid. A sample of the monobarium salt, kindly supplied by Dr S. Ratner, was treated with K_2SO_4.

Fructose-1-phosphate. A sample of the barium salt, kindly supplied by Dr R. K. Morton, was treated with K_2SO_4.

Phosphopyruvic acid. A solution was kindly supplied by Dr R. K. Morton.

Fructose-1:6-diphosphate. A commercial sample of the barium salt was brought into solution with HCl and treated with K_2SO_4. The solution contained much inorganic phosphate (25% of the organic phosphorus).

Analytical methods

Inorganic P *content of acid-labile compounds.* This was determined by the method of Berenblum & Chain (1938).

Total P. This was determined by (*a*) digestion with 10 N-H_2SO_4 (LePage, 1949*b*) followed by determination of inorganic P by the method of Lohmann & Jendrassik (1926), or (*b*) incubation with highly purified phosphomonoesterase (kindly supplied by Dr R. K. Morton). At the end of the incubation, the inorganic P was determined by the method of Lohmann & Jendrassik (1926), and the residual phosphorylated sugar was determined by the method described

in this paper. The values for the organic P have been corrected for the small amount of unhydrolysed ester (amounting to 0·5–2·5% of the total).

Acid-labile P. This was determined after treatment with N-HCl for 10 min. at 100°.

Total fructosephosphate. This was determined colorimetrically by Roe's (1934) method, using fructose as standard, on the assumption that fructosemonophosphate gives 79% of the colour given by an equivalent amount of fructose under these conditions (Slein, 1950).

Total adenosine. This was determined by measuring the optical density at 260 mμ. at neutral pH and converting to concentration by means of the value of $1 \cdot 59 \times 10^4$ obtained for the molar extinction coefficient of adenylic acid and ATP by Kalckar (1947).

Description of method

The description which follows is for the separate estimation of HDP, HMP, ATP and ADP in a mixture, assuming that no interfering materials are present. Methods of overcoming certain interfering substances will be described later. Separate portions of the reaction mixture are subjected to procedures *A*, *B* (*a*), *B* (*b*) and *C*.

Procedure A. Three spectrophotometer cells (1 cm.) are filled as follows:

	1 (ml.)	2 (ml.)	3 (ml.)
Glycylglycine, 0·25M, pH 7·6	0·3	0·3	0·3
Reduced DPN, 8×10^{-4}M approx.	—	0·3	0·3
Unknown solution	—	—	x
Water	2·4	2·1	$2 \cdot 1 - x$

After thorough mixing, the optical densities at 340 mμ. of cells 2 and 3 are measured, using cell 1 as reference. These readings are multiplied by the factor 2·7/3·0 to give D_2 and D_3 respectively. Immediately (zero time), 0·3 ml. of rabbit-muscle fraction *A* is added to each cell, the solutions are well

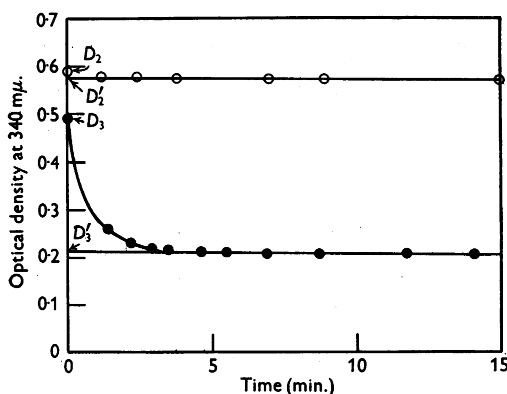

Fig. 1. Determination of hexosediphosphate by procedure *A*. 0·1 has been subtracted from all optical densities in the lower curve for ease of presentation. For description, see text.

mixed and readings taken against time for 10–20 min. Since the muscle fraction absorbs at 340 mμ., due to the presence of haematin compounds, the 0·3 ml. should be accurately pipetted into each cell. A typical measurement is shown in Fig. 1. It can be seen that cell 2 showed an immediate slight drop, followed by a very slow uniform decrease of the optical density. The immediate sudden drop is due to traces of substrates in the enzyme or reduced DPN preparations and is a blank to be applied to subsequent measurements. The slow uniform decline is probably due to autoxidizable flavoprotein in the enzyme preparation; in some preparations it is hardly detectable. The blank reading (D_2') is obtained by the extrapolation shown, which can be done very accurately. Cell 3 showed a fall of optical density at a decreasing rate until, after 4 min., the slow uniform decline shown by cell 2 was obtained. D_3' is obtained by extrapolation. The amount of HDP in the sample taken is given by $0 \cdot 241 \{(D_3 - D_3') - (D_2 - D_2')\}$. The factor 0·241 was calculated from the extinction coefficient of reduced DPN (Horecker & Kornberg, 1948) and reaction (*A*). The blank reading need be determined only once each day and, with a four-compartment cell holder, it is possible to determine three unknown solutions simultaneously. The volume of unknown solution used should preferably contain between 0·03 and 0·08 μmole HDP. Tri(hydroxymethyl)aminomethane may be used as buffer in place of glycylglycine.

Procedure B (*a*). This differs from procedure *A* in the following respects. (i) In addition to the substances previously mentioned, the cells contain $MgCl_2$ (7 μmoles), ATP (0·25 μmole) and sufficient water to make the final volume 2·6 ml. D_2 and D_3 are obtained by multiplying the initial readings by 2·6/3·0. (ii) Immediately before adding the rabbit-muscle fraction *A*, 0·1 ml. of fraction *B* is added to each cell. Otherwise, the procedure and calculations are the same as above. Time is saved by preparing a stock solution containing the glycylglycine, $MgCl_2$, reduced DPN and ATP, which can be stored at −15° for 1–2 weeks.

Procedure *B* (*a*) estimates the HDP + HMP content. The HMP content is given by the difference between the values obtained by procedures *B* (*a*) and *A*.

Procedure B (*b*). This is the same as procedure *B* (*a*), except that hexosemonophosphate (synthetic glucose-6-phosphate is very suitable, since it does not contain any HDP) replaces ATP. The blank ($D_2 - D_2'$) is often somewhat greater in this measurement, since many samples of DPN contain some ~P. Procedure *B* (*b*) measures the HDP + ~P content. The ~P content is given by the difference between the values obtained by procedures *B* (*b*) and *A*.

Procedure C. To the solution to be analysed are added 0·3 ml. 0·5M-phosphate buffer, pH 7·3; 0·1 ml. 0·25M-glucose; 0·1 ml. M-NaF and water to make the final volume 2·8 ml. Hexokinase (0·1 ml.) and 0·1 ml. 0·15M-$MgCl_2$ are added and, when the reaction is completed, 0·5 ml. 40% (w/v) trichloroacetic acid is added and the mixture centrifuged. The time required depends upon the activity of the hexokinase and should be determined in a separate experiment. An example with very dilute hexokinase is given in Table 1. If too long a period is allowed to elapse after complete reaction the final value may be slightly low due to the presence of a trace of phosphatase in some hexokinase preparations. A known volume (usually 2 ml.) of the supernatant is neutralized with N-KOH. A suitable sample of this solution is then analysed by procedure *B* (*a*). After multiplication by the various dilution factors, this gives the amount of ATP in the original solution. The amount of ADP equals the ~P, determined above, minus twice the ATP content.

For the complete analysis of a mixture of HDP, HMP, ATP and ADP as described above, rabbit-muscle fraction *A*

must contain very little phosphohexokinase. If phosphohexokinase is present, procedure *A* will give not HDP but HDP + HMP or HDP + ~P, whichever is the lower. Some preparations of rabbit-muscle fraction *A* contain sufficient phosphohexokinase to interfere and these preparations are unsuitable if both HDP and ~P or HMP are present. Dr R. K. Morton (private communication) has found that filtering fraction *A* through a pad of Super-Cel removed the phosphohexokinase almost completely.

Table 1. *Determination of ATP*

(0·1 ml. of stock solution of ATP treated by procedure *C* for different periods; 0·01 ml. crude hexokinase (0·5 mg. protein) used.)

Time (min.)	HMP found (μmoles)
2	1·02
4	1·38
10	1·49
20	1·50
60	1·54

EXAMINATION OF METHOD

Effect of glyceraldehydephosphate dehydrogenase in the enzyme mixture

If glyceraldehydephosphate dehydrogenase is present in the rabbit-muscle enzyme preparations, one might expect that part of the phosphoglyceraldehyde would reduce the DPN and thereby seriously interfere with the method described.

(7) Glyceraldehydephosphate + DPN + H_3PO_4 → diphosphoglyceric acid + reduced DPN (*glyceraldehydephosphate dehydrogenase*).

To test whether such interference was occurring under the conditions of the procedure described,

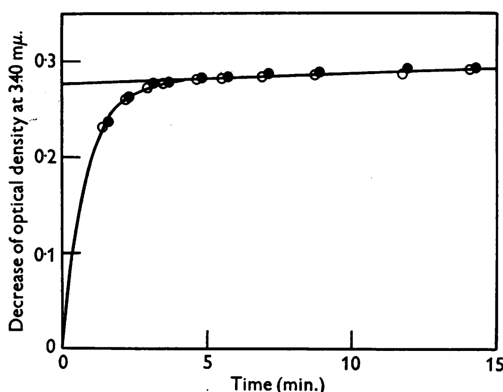

Fig. 2. Effect of iodoacetate on estimation of hexosemonophosphate by procedure *B* (*a*). ○, usual reaction mixture; ●, 0·001 M-iodoacetate added.

iodoacetate (0·001 M) was added to inhibit the glyceraldehydephosphate dehydrogenase. Cori, Slein & Cori (1948) have demonstrated that, under comparable conditions, 4×10^{-4} M-iodoacetate was sufficient completely to inhibit reaction (7). Fig. 2 shows that 0·001 M-iodoacetate caused no difference in the rate of oxidation of reduced DPN or in the amount oxidized. It follows that reaction (7) must be proceeding at an insignificant rate compared with reaction (3), a conclusion which is supported by the fact that the addition of inorganic phosphate or arsenate, which would be expected to increase the rate of reduction of DPN by glyceraldehydephosphate, did not affect the overall reactions studied. Reaction (3) is probably much faster than reaction (7), even in the presence of glyceraldehydephosphate dehydrogenase (Racker, 1947) and most of the latter enzyme remains in the supernatant after precipitation of rabbit-muscle fraction *A*.

Interfering substances

These are of two types: (*a*) those which oxidize reduced DPN rapidly in the presence of the muscle enzymes and are therefore erroneously included with the substance being analysed, and (*b*) those which react slowly with reduced DPN and therefore increase the rate of the slow decline after the oxidation of reduced DPN by the dihydroxyacetonephosphate is complete.

(*a*) The only substances of the first type which interfere with the estimation of HDP or HMP are pyruvate (or substances yielding pyruvate, namely phosphopyruvate and phosphoglycerate, see below) and oxaloacetate. The rabbit-muscle enzymes contain lactic and malic dehydrogenases which catalyse reactions (8) and (9) respectively

(8) Pyruvate + reduced DPN → lactate + DPN (*lactic dehydrogenase*)

(9) Oxaloacetate + reduced DPN → malate + DPN (*malic dehydrogenase*).

Pyruvate is readily determined by means of purified lactic dehydrogenase. Fig. 3 shows the separate determination of pyruvate and HDP in a mixture by the successive addition of lactic dehydrogenase and rabbit-muscle fraction *A*. Oxaloacetate may be determined by malic dehydrogenase (Straub, 1942) or after decarboxylation to pyruvate. The lactic dehydrogenase preparation described under Experimental contained sufficient malic dehydrogenase so that, in a suitable dilution, pyruvate and oxaloacetate could be separately determined, the pyruvate oxidizing the reduced DPN rapidly and oxaloacetate more slowly. Care is necessary if triosephosphates are present in the solution to be analysed, since some preparations of lactic dehydrogenase contain the enzymes necessary for reactions (2) and (3) in small concentrations, although quite free of aldolase.

Pyruvate and oxaloacetate also interfere with the determination of ~P. Another substance which also interferes with this measurement is creatine-

phosphate, since creatinephosphokinase, the enzyme catalysing reaction (10), is present in the preparations.

(10) Creatinephosphate + ADP → creatine + ATP (*creatinephosphokinase*).

70 % of creatinephosphate reacted in this way in 21 min., in one measurement. If creatinephosphate is present additional creatinephosphokinase should be added, so that reaction (10) proceeds to completion before the ADP is all used up by reaction (6) followed by reaction (5). The ~P analysis will then include creatinephosphate as well as the reactive groups of ATP and ADP. The addition of purified creatinephosphokinase + 0·01 μmole of ADP has, in fact, proved to be a satisfactory method of estimating creatinephosphate (Morton, 1952; Kratzing & Narayanaswami, 1953). An alternative method of estimating creatinephosphate is to add creatinephosphokinase and ADP to the components of procedure *C*. In this case, however, careful timing of the reaction is necessary owing to the presence of a little phosphatase in the creatinephosphokinase preparation. Since the hexokinase preparations are free from creatinephosphokinase, creatinephosphate does not interfere with the estimation of ATP.

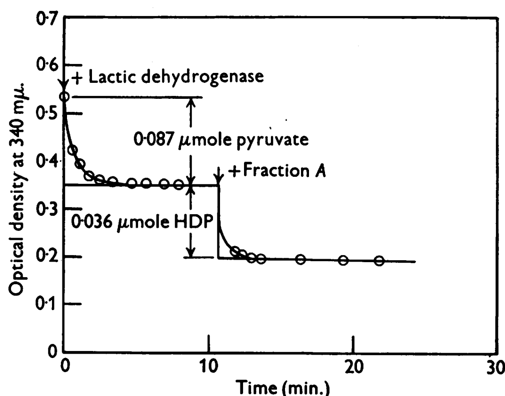

Fig. 3. The determination of pyruvate and hexosediphosphate on the same solution by the successive addition of lactic dehydrogenase and rabbit-muscle fraction *A*. Experimentally determined optical densities have been corrected for the dilution caused by the various additions.

Phosphopyruvate, which is also an energy-rich phosphate compound, and phosphoglycerate, which can be considered as a potentially energy-rich phosphate compound, are also estimated as ~P, since the enzymes catalysing reactions (11) and (12) are present in the rabbit-muscle preparations.

(11) 3-Phosphoglycerate ⇌ 2-phosphoglycerate ⇌ phosphopyruvate (*phosphoglyceromutase and enolase*),

(12) Phosphopyruvate + ADP → ATP + pyruvate (*pyruvic phosphokinase*).

Since the pyruvate formed by reaction (12) will oxidize one molecule of reduced DPN (see above), each molecule of phosphoglycerate or phosphopyruvate will oxidize three molecules of reduced DPN. Thus the total ~P value will be somewhat overestimated if these substances are present in appreciable amounts. This is, however, usually unlikely. The method is a very sensitive procedure for the estimation of phosphoglycerate and phosphopyruvate, in the absence of other ~P compounds. Thus a solution of phosphopyruvate containing 2·02 μmoles total P/ml. gave on analysis 1·91 μmoles/ml. by the enzymic method assuming that 1 mol. phosphopyruvate oxidized 3 mol. reduced DPN; similarly, a solution of phosphoglyceric acid containing 8·4 μmoles total P/ml. gave 7·9 μmoles/ml. by the enzymic method. 1:3-Diphosphoglyceric acid has not been tested, but probably both phosphate groups will be estimated as ~P. Phosphopyruvate and phosphoglycerate will interfere in the same way as pyruvate in the estimation of HDP or HMP, since they are rapidly dephosphorylated in the presence of the rabbit enzymes, even in the absence of ADP. Possibly adenylic acid, present as an impurity in the DPN, is phosphorylated (maybe indirectly) with sufficient speed, although this reaction is very much slower than the phosphorylation of ADP.

(*b*) Substances which react slowly with reduced DPN include:

(i) α-Ketoglutarate (reaction 13)

(13) α-Ketoglutarate + NH_3 + reduced DPN → glutamate + DPN (*glutamic dehydrogenase*).

NH_3 is supplied by the $(NH_4)_2SO_4$ introduced with the enzyme preparations. The amount of glutamic dehydrogenase varies considerably from preparation to preparation.

(ii) Fructose-1-phosphate (procedures *B* (*a*) and *C* only) which is slowly phosphorylated by ATP to HDP by reaction (14).

(14) Fructose-1-phosphate + ATP → HDP + ADP.

(iii) High concentrations of glucose and fructose. These may react because of traces of hexokinase and fructokinase in the rabbit-muscle preparations. Provided sufficient phosphohexokinase is present, hexokinase and fructokinase will not interfere with the estimation of ~P.

(iv) High concentrations of glycogen in the presence of inorganic phosphate slowly liberate glucose-1-phosphate by the action of phosphorylase.

Substances which react slowly with reduced DPN do not affect the value of HDP, HMP or ~P obtained by the extrapolation; they only affect the slope of the line which is extrapolated. This is shown in the case of α-ketoglutarate in Fig. 4; 0·0495 μmole of HDP was found in the absence of α-ketoglutarate, while 0·0475 μmole was found in the presence of

5 μmoles α-ketoglutarate. The effect of fructose-1-phosphate is shown in Fig. 5. In this experiment, considerably more enzyme was added than usual (hence the high blank value). Nevertheless, the rate of oxidation of reduced DPN by as much as 0·6 μmole fructose-1-phosphate is not so great as to make the extrapolation impossible. Under the same conditions, all the fructose-6-phosphate was reduced in less than 2 min. It is clear that fructose-6-phosphate reacts very much more rapidly than fructose-1-phosphate in the presence of the rabbit-muscle fractions. Slein, Cori & Cori (1950) have shown that different enzymes are involved in the phosphorylation of these two compounds by muscle.

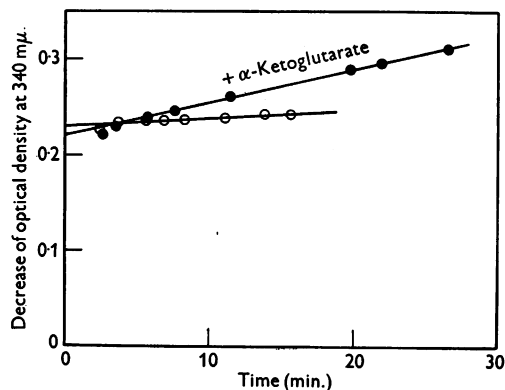

Fig. 4. Effect of α-ketoglutarate (5 μmoles) on the determination of hexosemonophosphate.

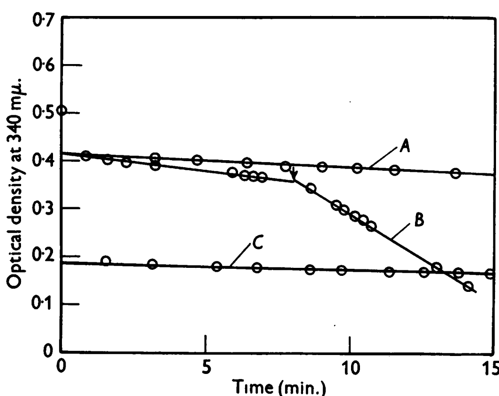

Fig. 5. Effect of fructose-1-phosphate. Curve A shows a blank determination without any added fructose-1-phosphate. Curve B was obtained when 0·1 μmole fructose-1-phosphate was initially present and a further 0·5 μmole was added at the arrow. The experimentally determined values of optical densities have been corrected for the dilution caused by this addition. Curve C shows the effect of 0·055 μmole glucose-6-phosphate without fructose-1-phosphate.

High concentrations of trichloroacetate considerably inhibit the enzymes and if the amounts of phosphorylated sugar or ~P are so small that a large sample of the acid extract must be used for analysis, it is preferable to deproteinize with perchloric acid, neutralize with 4 N-KOH, cool to 0° and remove the potassium perchlorate by filtration (I am indebted to Dr C. C. Kratzing for this suggestion).

The following substances have been found to have no effect on the measurements—inorganic phosphate (unless the concentration is sufficient to precipitate magnesium), arsenate, malonate, fluoride, muscle adenylic acid, succinate, acetate, ethanol, TPN, DPN, ascorbic acid, inorganic pyrophosphate, ribose-5-phosphate. A large amount of cyanide (10 μmoles) decreased the value by about 10 %. The lack of effect with fluoride shows that phosphatases are not causing any losses.

Reproducibility of the method

The reproducibility of the method for estimating HDP or HMP is excellent. The molarity of a solution of HDP, stored in the frozen state, was found to be 0·0352, 0·0340, 0·0336, 0·0360 and 0·0348 in successive analyses on different days, spread over a period of 3 months. The molarity of a solution of fructose-6-phosphate was similarly found to be 0·0338, 0·0331, 0·0326, 0·0332, 0·0331 in successive analyses.

Analyses of stock solutions of phosphorylated sugars

In Table 2 are reported analyses of stock solutions of various phosphorylated sugars, both by the present method and by determination of organic P. The latter was determined in two ways, (*a*) by acid digestion, and (*b*) by hydrolysis with purified phosphomonoesterase. All three methods gave excellent agreement in the case of enzymically prepared hexosemonophosphate (probably the equilibrium mixture of glucose-6-phosphate and fructose 6-phosphate) and fructose-1:6-diphosphate. In the case of glucose-1-phosphate, which was not determined by the phosphomonoesterase method, agreement between acid digestion and the enzymic method was also very close. The two other samples showed some disagreements which were further examined.

Both methods of estimating the organic P content of the solution of fructose-6-phosphate agreed and this was also in reasonable agreement with fructose-6-phosphate determined colorimetrically. The enzymic procedure described in this paper, however, gave values approximately 17 % lower. The most likely explanation of this discrepancy is the presence of fructose-1-phosphate in the sample of fructose-6-phosphate, which was prepared commercially,

Table 2. *Analyses of stock solutions of phosphorylated sugars*

(Methods of preparation of stock solutions and analytical methods are described in the Experimental section. All values are given as μmoles/ml.)

Compound	Total P − inorganic P: Acid digestion	Total P − inorganic P: Phosphomono-esterase	Phosphorylated sugar measured by enzymic method
Glucose-1-phosphate*	9·8	—	9·6
Hexosemonophosphate (enzymic)	17·0	16·5	16·9
Fructose-6-phosphate†	40·5	40·1	33·2‡
Glucose-6-phosphate	46·0	41·0	37·3
Fructose-1:6-diphosphate	21·2	20·7	19·9

* 9·9 μmoles/ml. calculated from weight of crystals.
† 41·9 μmoles/ml. measured colorimetrically (Roe, 1934).
‡ Including 0·28 μmole/ml. HDP.

Table 3. *Partial hydrolysis of synthetic glucose-6-phosphate by phosphomonoesterase*

(A very dilute preparation of highly purified phosphomonoesterase was used; the erratic course of the hydrolysis/time curve is probably due to different degrees of surface inactivation in different tubes.)

Time of hydrolysis (hr.)	Inorganic P (μatoms)	ΔP (μatoms)	Glucose-6-phosphate (μmoles)	Δ Glucose-6-phosphate (μmoles)
0	0·01	—	1·86	—
0·17	0·01	0	1·88	0·02
0·5	0·55	0·54	1·23	−0·63
1	0·60	0·59	1·25	−0·61
3	1·04	1·03	0·83	−1·03
36	2·01	2·00	0·04	−1·82

Table 4. *Analyses of solutions of ATP and ADP*

(All concentrations are expressed as μmoles/ml.)

	Commercial ATP	Laboratory ATP				Laboratory ADP		
Inorganic P	11·6	1·7	1·6	1·7	—	0·8	—	—
ATP	6·6	12·4	23·9	19·3	21·7	0	0	—
~P	19·9	25·1	48·5	39·0	48·9	6·8	19·1	20·9
Acid-labile P	25·7	28·5	51·4	42·4	—	7·4	—	—
ADP*	6·7	0·3	0·7	0·4	5·5	6·8	19·1	20·9
ATP + ADP	13·3	12·7	24·6	19·7	27·2	6·8	19·1	20·9
Total adenosine	12·9	14·2	25·4	19·8	26·9	6·6	19·0	21·4

* Calculated (~P − 2 ATP).

probably by acid hydrolysis of fructose-1:6-diphosphate. An acid hydrolysis curve revealed the existence of a small amount of a more rapidly hydrolysable component, which is in agreement with this hypothesis. Fructose-1-phosphate would be indistinguishable from fructose-6-phosphate by all the methods used in Table 2, except the enzymic procedure.

The HMP content of the sample of synthetic glucose-6-phosphate was only 81 % of that expected from the organic P content. The phosphate liberated by the phosphomonoesterase was, however, considerably less than the total organic P. Table 3 shows close agreement between HMP disappearing and inorganic P appearing after partial hydrolysis of the preparation. It appears that the glucose-6-phosphate preparation contains an organic phosphorus compound which does not react with the rabbit-muscle enzymes and is only partially hydrolysable by phosphomonoesterase. Ochoa *et al.* (1950) found that only 80 % of a sample of synthetic glucose-6-phosphate (presumably standardized by total P determination) reduced TPN in the presence of glucose-6-phosphate dehydrogenase.

Analyses of solutions of ATP and ADP

Table 4 shows analyses of solutions prepared from samples of ATP obtained commercially and of ATP and ADP prepared in the laboratory. In all samples, except one, the ATP + ADP agreed closely with the total adenosine content calculated from the absorption at 260 mμ. Most laboratory-made ATP preparations contained practically no ADP (in the case of the exception, treatment with trichloroacetic acid was unduly delayed after the death of the animal). The commercial sample of ATP contained

as much ADP as ATP, as well as large amounts of inorganic P. The acid-labile P in all cases exceeded the ~P determined enzymically, the discrepancy being considerable only in the case of the commercial ATP. The discrepancy is probably due to pyrophosphate (Bailey, 1949).

Table 5. *Analysis of ADP prepared by Bielschowsky's method*

	μmoles/ml.
Inorganic P	4·1
ATP	4·3
~P	18·1
ADP*	9·5
ATP + ADP	13·8
Total adenosine	20·3
Acid-labile P	18·1
Acid-labile P/total adenosine	0·90

* Calculated (~P − 2 ATP).

The method of Bielschowsky (1950) for preparing ADP (hydrolysis at pH 4·5, in the presence of Mg^{++}) was followed, using the commercial sample of ATP, which already contained as much ADP as ATP. The analysis of the final product is given in Table 5. Although the ratio of acid-labile P to total adenosine is close to the theoretical for ADP (1·0), the product contains considerable ATP and much material absorbing at 260 mμ. not accountable in terms of ATP + ADP. This latter material is very likely adenylic acid. Thus, even though all the ATP is not hydrolysed, much of the ADP has been further hydrolysed to adenylic acid. These results give little reason to hope that ADP can be satisfactorily prepared from ATP by controlled acid hydrolysis. A weakness of the conventional chemical methods for following such reactions is that a mixture of equimolar proportions of ATP and adenylic acid gives the same result as pure ADP. The agreement between the ~P and acid-labile P in Table 5 suggests that all the pyrophosphate originally present in the commercial ATP has been removed.

DISCUSSION

The methods described in this paper have proved very useful for a number of investigations. In common with similar procedures developed by Ochoa and his associates, they combine the advantages of the specificity obtained by using enzymes with the sensitivity of spectrophotometric methods. Although a few compounds do interfere, none of these has actually been present in any of the problems investigated by these methods. The specificity of enzymic methods gives them the very great advantage over chemical methods that physical separations of different phosphorus compounds are not necessary. They are particularly valuable for the estimation of small amounts of phosphorylated sugars or adenine nucleotides in the presence of large amounts of inorganic phosphate. Inorganic phosphate must be removed before application of chemical methods to organic phosphorus compounds, and when there is a large excess of inorganic phosphate it cannot be precipitated without large losses of organic phosphate by co-precipitation (Lehninger, 1949; Ennor & Rosenberg, 1952).

The methods described in the present paper determine in a mixture (*a*) total hexosemonophosphate, (*b*) hexosediphosphate + triosephosphates, (*c*) ATP and (*d*) ~P. No attempt has been made to measure the individual hexosemonophosphates separately or to distinguish hexosediphosphate from triosephosphate. In oxidative phosphorylation experiments, for example, the esterified phosphate is often transferred to glucose by means of hexokinase. Since yeast hexokinase preparations contain the active hexosemonophosphate isomerase, the esterified phosphate will appear as both glucose-6-phosphate and fructose-6-phosphate and the total hexosemonophosphate found will correctly measure the esterification of inorganic phosphate. If separate determinations are required, this could probably be achieved either by purification of the rabbit-muscle enzymes or by physical separation of the esters, for example by paper chromatography (Hanes & Isherwood, 1949).

The method described is not the only enzymic method available for the estimation of hexosemonophosphate. Ochoa *et al.* (1950), Slein (1950) and Kornberg & Pricer (1951*b*) have used the reduction of TPN by glucose-6-phosphate, catalysed by glucose-6-phosphate dehydroganase. Slein (1950) determined glucose-6-phosphate + fructose-6-phosphate by addition of hexosephosphate isomerase. By the further addition of phosphomannose isomerase the method becomes specific for mannose-6-phosphate. Mannose-6-phosphate has not been tested in the present work, but since Slein (1950) found that phosphomannose isomerase was precipitated from rabbit-muscle extract between 0·45 and 0·55 saturation with ammonium sulphate, it is very likely that this enzyme is present in the rabbit-muscle fractions used in the present paper and that mannose-6-phosphate, if present, would be estimated with the other hexosemonophosphates. Kornberg & Pricer (1951*b*) have recently adapted the method using glucose-6-phosphate dehydrogenase to the determination of ATP and ADP by the separate addition of hexokinase and myokinase. No special advantages of the present procedure over the methods used by the above authors is claimed. Which method is used will be determined by convenience and the interfering substances likely to be present. Since, in the present method, each molecule

of hexosemonophosphate reacts with two molecules of reduced DPN whereas only one molecule of TPN is reduced by glucose-6-phosphate, the former method has double the sensitivity. This will not often be an advantage, because both methods are highly sensitive, but was an important consideration in choosing the present method for studying phosphorylation coupled with the reduction of cytochrome *c* (Slater, 1950).

Vishniac & Ochoa (1952) have recently determined HDP by the reduction of DPN catalysed by purified aldolase, triosephosphate isomerase, glyceraldehydephosphate dehydrogenase, ADP and the enzyme which catalyses the phosphorylation of ADP by diphosphoglyceric acid. Special care must be taken to remove glycerolphosphate dehydrogenase from the enzymes if this method is used (Racker, 1947) and it is not as convenient for most purposes as the method described in this paper. Vishniac & Ochoa's (1952) special problem was to estimate small quantities of HDP in the presence of large amounts of 3-phosphoglyceric acid, which interferes with the present method.

Kornberg & Pricer (1951*b*) have also introduced a more specific method for the estimation of ADP by the use of phosphoenol pyruvic acid and pyruvic phosphokinase in the presence of lactic dehydrogenase and reduced DPN. According to these authors, ADP is the specific acceptor and, for every molecule present, one molecule of pyruvate appears and oxidizes the equivalent amount of reduced DPN.

The sensitivity of the enzymic methods is such as to lend them to the estimation of adenine nucleotides in very small amounts of biological materials. The optimal amount of ATP to be taken for the estimation is only 0·025 μmole in 3 ml., and 0·01 μmole can be determined with considerable accuracy. If microcells were used, this figure could be greatly decreased.

SUMMARY

1. An enzymic method for the determination of phosphorylated sugars and energy-rich compounds is described. The method depends upon the enzymic conversion of these compounds to dihydroxyacetonephosphate, which then reacts with reduced DPN in the presence of glycerolphosphate dehydrogenase. The amount of reduced DPN reacting is determined spectrophotometrically.

2. The method is highly sensitive, 0·05 μmole of phosphorylated sugar or energy-rich phosphate being measured with an accuracy of a few per cent.

3. In a complex mixture separate analyses are obtained for (*a*) hexosediphosphate + triosephosphates, (*b*) hexosemonophosphates (glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, but not fructose-1-phosphate), (*c*) ATP and (*d*) other energy-rich compounds (ADP, creatinephosphate, phosphopyruvate (which is over-estimated by 50 %)).

4. The only substances which interfere are (i) pyruvate and oxaloacetate, each molecule of which reacts as one-half a molecule of hexosediphosphate and (ii) phosphoglycerate, which behaves like phosphopyruvate. Pyruvate and oxaloacetate may be separately determined with lactic and malic dehydrogenases.

5. Analyses of preparations of phosphorylated sugars and adenine nucleotides, either prepared in the laboratory or obtained commercially, have been made both by the new method and by conventional chemical methods. Agreement was very close in most cases. Where there was disagreement this has been traced to the presence in the preparations of impurities which are estimated by the chemical but not by the enzymic method.

I wish to thank Dr E. Racker, who made the initial suggestion leading to this study, Prof. S. Ochoa, in whose laboratory the work commenced, Prof. D. Keilin, F.R.S., in whose laboratory it was continued, and Dr S. Korkes, for their advice. I am also indebted to a number of people for the supply of various materials, acknowledged in the text. Finally, I wish to acknowledge with thanks the receipt of Fellowships from the Rockefeller Foundation and the Australian National University and a personal grant from the Agricultural Research Council during different stages of this work.

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The Effect of Thiol and other Group-specific Reagents on Erythrocyte and Plasma Cholinesterases

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(*Received* 17 *May* 1952)

That the activity of certain enzymes may depend on the presence in the enzyme molecule of intact sulphydryl groups is now widely accepted and the cholinesterases have been considered to belong to this class of 'sulphydryl enzymes' (Nachmansohn & Lederer, 1939; Barron & Singer, 1943; Stadie, Riggs & Haugaard, 1945; Thompson, 1948). Many of the relevant observations were, however, made before it was realized that cholinesterases from different sources are not identical in specificity and other properties (Alles & Hawes, 1940; Richter & Croft, 1942; Mendel & Rudney, 1943; Zeller & Bissegger, 1943), and a critical survey of the literature reveals that the evidence for regarding any one cholinesterase as an —SH enzyme rests almost entirely upon inhibition by a few reagents, some by no means specific for —SH groups. The present work was undertaken with the object of investigating the possible sulphydryl nature of the cholinesterases of the human plasma and erythrocytes and attempting to decide whether sulphydryl groups can be regarded as having functional significance in relation to the activity of these representative mammalian cholinesterases.

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Table 1 summarizes existing work with —SH inhibitors. The most extensive observations are those of Nachmansohn & Lederer (1939) who found that the cholinesterase of the electric organ of the torpedo was inhibited by such typical thiol reagents as copper, maleic acid, iodoacetate, oxidized glutathione and alloxan. They concluded that torpedo cholinesterase was an —SH enzyme, though with some of these reagents rather large concentrations and prolonged incubation periods were required to produce a significant inhibition. Moreover, the specificity of some of these reagents is doubtful, while other thiol reagents, such as arsenite, were not tested. Mapharside (3-amino-4-hydroxyphenylarsenoxide) was, however, found by Barron & Singer (1943) to be fairly powerful as an inhibitor of a cholinesterase of unspecified origin (electric-organ cholinesterase) while Thompson (1947) found that pigeon-brain cholinesterase was fairly sensitive to arsenite. Thompson (1948) also reported that pigeon-brain cholinesterase, unlike pigeon-brain 'pyruvate oxidase', could be protected from arsenite by a monothiol, cysteine ester hydrochloride, as well as by the dithiol 2:3-dimercaptopropanol (BAL). He tentatively suggested that cholinesterase is a monothiol enzyme, that is, its activity possibly depends on the presence of one thiol group per active centre in contrast to the 'dithiol enzyme' of pyruvate oxidation which is presumed to form an arsenic complex of stability intermediate between those which arsenic forms with cysteine and with BAL.

Horse serum cholinesterase was found by Massart & Dufait (1939) to be inhibited by arsenite in fairly high concentration. Mackworth (1948) found that this enzyme is not markedly sensitive, as are succinic and triosephosphate dehydrogenases, activated papain and other typical —SH