

the aglycone $C_{20}H_{14}O_4$ of the plant pigment haemocorin, the only other known perinaphthenone from natural sources. This requires the coupling of one acetate and two shikimic acid-derived C_6-C_3 units and involves an intermediate related to the plant product *p*-hydroxycinnamoylferuloylmethane ($C_{20}H_{18}O_5$), which then undergoes cyclization.

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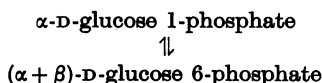
Equilibrium Constant of Phosphoryl Transfer from Adenosine Triphosphate to Galactose in the Presence of Galactokinase

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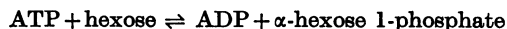
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The free energy of hydrolysis of adenosine triphosphate has been calculated as the sum of the free energies of phosphoryl transfer from this compound to $C_{(6)}$ of D-glucose and from $(\alpha + \beta)$ -D-glucose 6-phosphate to water (Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957*a, b*; Robbins & Boyer, 1957). The equilibrium constant of the first reaction, catalysed by hexokinase, is so large that accurate estimation of the minor components of the equilibrium mixture is difficult. Colowick & Sutherland (1942) found the equilibrium constant of the phosphoglucomutase reaction,



to be 17 at pH 7.5. An α -hexose 1-phosphate thus has a more negative free energy of hydrolysis than

the corresponding hexose 6-phosphate, and the equilibrium constant of the reaction



is correspondingly smaller than that of the hexokinase reaction. Galactokinase catalyses formation of α -D-galactose 1-phosphate from adenosine triphosphate and D-galactose (Trucco, Caputto, Leloir & Mittelman, 1948; Wilkinson, 1949). It seemed probable that at pH 7.0 and 25°, in the presence of more than 10 mM-Mg²⁺, the equilibrium constant of the galactokinase reaction would be more than 10 and less than 100.

Addition of adenylate kinase to a solution containing adenosine diphosphate and adenosine triphosphate catalyses formation of adenosine monophosphate, and ensures that



is about 2-3 at pH 7.0 and 25° in the presence of more than 10 mM-Mg²⁺ (cf. Kalckar, 1943; Eggleston & Hems, 1952; Bowen & Kerwin, 1954). By selection of a suitable ratio of total adenine derivative to total galactose derivative, the ratio of triphosphate to diphosphate may be kept in a convenient range through the coupled action of galactokinase and adenylate kinase.

In the work described here, mixtures of adenosine triphosphate and D-galactose or adenosine diphosphate and α -D-galactose 1-phosphate have been brought to equilibrium with this pair of enzymes and the equilibrium constant has been measured. The value so obtained has been used in calculation of the free energy of hydrolysis of adenosine triphosphate (Atkinson, Johnson & Morton, 1959). Combination of the equilibrium constants with published values has afforded a consistent set of free energies of hydrolysis of many phosphorylated metabolites (Atkinson & Morton, 1960 and unpublished results).

MATERIALS

Chemicals

Perchloric acid, trichloroacetic acid, formic acid and citric acid were A.R. grade (British Drug Houses Ltd.). MgCl₂ (A.R.; British Drug Houses Ltd.) was standardized by potentiometric determination of chloride. Diethyl ether was washed with 0.1N-NaOH, distilled and freed of peroxides with activated alumina.

D-Galactose. D-Galactose (Thomas Kerfoot Ltd., Vale of Bardsley, Lancs.) was dried to constant weight at 90° and had $[\alpha]_D^{20} + 80.4 \pm 0.5^\circ$ in water after 24 hr. (c, 2.0) (Found: C, 39.7; H, 6.7; O, 53.3. Calc. for C₆H₁₂O₆: C, 40.0; H, 6.7; O, 53.3). A single component was detected with AgNO₃ and alkali after chromatography in ethyl acetate-pyridine-water (2:1:1, by vol.). Analysis with hexokinase and glucose 6-phosphate dehydrogenase showed the presence of 0.8% of glucose.

α -D-Galactose 1-phosphate. (i) Material used in equilibrium studies was prepared from D-galactose (Hansen, Rutter & Krichevsky, 1955). After isolation of the potassium salt as described for potassium glucose 1-phosphate (Krahl & Cori, 1949) the ester was further purified by elution from Dowex-2 (Cl⁻ form) with a gradient of NaCl in HCl (Isselbacher, 1958). Chromatography in propan-2-ol-aq. 10N-NH₃ soln. (5:3, v/v) showed the presence of about 10% of orthophosphate, the only contaminating phosphate. A solution of the most concentrated eluate fractions had $[\alpha]_D^{20} + 0.43 \pm 0.01^\circ$ in a 10 cm. tube. After hydrolysis at 100° for 10 min. in 0.5N-perchloric acid it contained orthophosphate corresponding to 11.3 μ moles/ml. of original solution; the galactose content after hydrolysis in N-H₂SO₄ corresponded to 10.3 μ moles/ml. Calculated from galactose content, $[M]_D^{20}$ was +423 \pm 9°; from Wilkinson's (1949) results, $[M]_{5461}^{20}$ was calculated to be +435° for the barium salt of α -D-galactose 1-phosphate prepared enzymically. Kosterlitz (1943) reported the same rotation for ester prepared by a non-enzymic method.

(ii) The same phosphate ester was isolated from galactokinase equilibrium mixtures. Protein was removed by

addition of 0.05 vol. of perchloric acid (72%, v/v) and filtration at 0°. The filtrate was brought to pH 8.6 with 10N-KOH and centrifuged. Barium acetate (5%, w/v; 12 ml.) was added to 110 ml. of the supernatant, the pH was adjusted to 9.0 with saturated Ba(OH)₂ and 100 ml. of ethanol was added. The barium α -D-galactose 1-phosphate was purified as in (i) above, and showed identical behaviour on chromatography. A sample which had $[\alpha]_D^{20} + 0.13 \pm 0.01^\circ$ was hydrolysed as in (i) and then contained galactose and orthophosphate corresponding to 2.6 and 3.2 mM in the unhydrolysed sample. Chromatography as in (i) showed the presence of free orthophosphate in the ester. From the galactose ester content, $[M]_D^{20}$ was +500 \pm 40°. After removal of ionic material with Dowex-50 (H⁺ form) and Dowex-2 (acetate form) the sugar liberated by acid hydrolysis was chromatographed in ethyl acetate-pyridine-water (2:1:1, by vol.). The only reducing component detected coincided with authentic galactose.

Adenosine 5'-phosphate. On electrophoresis at pH 4.5 the sodium salt (Sigma Chemical Co.) gave a single component which absorbed at 254 m μ and accounted for 97% of the total absorption at 259 m μ . The adenosine 5'-phosphate (AMP) content by enzymic analysis (see Methods and Results section) was 97% of the total material absorbing at 259 m μ .

Adenosine diphosphate. The sodium salt (Sigma Chemical Co.) contained 90% of adenosine diphosphate (ADP) and 10% of adenosine triphosphate (ATP) by electrophoretic analysis. By enzymic analysis 89% of the total absorption at 259 m μ was due to ADP.

Adenosine triphosphate. The sodium salt (Sigma Chemical Co.) was used. A single component, detected by electrophoresis, accounted for 98% of the material absorbing at 259 m μ . Enzymic analyses for total reactive phosphoryl groups (\sim P; Slater, 1953) accounted for 99 \pm 1% of the total absorption.

Reduced diphosphopyridine nucleotide. This (reduced DPN) was prepared from oxidized DPN (98%; Sigma Chemical Co.) by reduction with ethanol and alcohol dehydrogenase (Rafter & Colowick, 1955). For use in estimation of ATP and ADP the alcohol dehydrogenase was inactivated by pouring the reaction mixture through a long-stemmed funnel into a tube preheated in boiling water. Within 2 min. the ethanol started to boil, and after a further 3 min. the solution was cooled and stored at -15°. Material prepared in this way contained traces of AMP. Reduced DPN for estimation of AMP was purified as its barium salt and freed of barium with K₂SO₄. No AMP was detected in this material.

Phosphoenolpyruvate. The barium silver salt (Sigma Chemical Co.; 100 mg.) was dissolved in 2.5 ml. of 0.2N-HNO₃ at 0°. After addition of 2N-H₂SO₄ (0.23 ml.) and removal of BaSO₄, AgCl was precipitated with 0.25 ml. of N-HCl and removed by centrifuging and filtration. After adjustment to pH 7 with 0.3 ml. of 2N-KOH the solution (about 3 ml.) contained 60 mM-phosphoenolpyruvate and 0.2 mM-pyruvate (estimated with lactate dehydrogenase).

Enzyme preparation

Galactokinase. This was prepared from yeast (*Saccharomyces fragilis*) adapted to galactose, which was generously supplied by the Sigma Chemical Co. The procedure described by Leloir & Trucco (1955) was modified by omission of the bentonite adsorption step. The enzyme was dialysed for

20 hr. against 0.01 M-cysteine at pH 7.0 and was freed of uridine diphosphate galactose 4-epimerase by heating for 2 min. at 50° (Maxwell, 1957). The solution contained 80 mg. of protein/ml. and catalysed phosphorylation of 0.25 μ mole of galactose/min./mg. of protein in a system containing 10 mM-MgCl₂, 10 mM-ATP and 8 mM-galactose at 25° and pH 7.0 (assayed by titration with NaOH).

The enzyme was transported from St Louis to Adelaide in closed tubes in solid CO₂ and was stored under similar conditions. It retained its activity for over 12 months.

The enzyme contained adenylate kinase and hexokinase and traces of adenylate deaminase and phosphatase.

Adenylate kinase. This was prepared from rabbit muscle as described by Colowick (1955).

Lactate dehydrogenase. The crystalline preparation from muscle (Sigma Chemical Co.) contained enough pyruvate kinase for estimation of ADP and related compounds (see Methods and Results section).

Muscle enzymes for assay of phosphoryl donors. The following modification of the procedure described by Slater (1953) was used. The alkaline extract of muscle was brought to 30% saturation at pH 7.8 with (NH₄)₂SO₄ and aq. NH₃ soln. and filtered through Hyflo Supercel. The filtrate was brought to 70% saturation at pH 7.8 and filtered through fluted Whatman no. 1 paper overnight at 3°. The precipitate on the paper was washed with 70% saturated (NH₄)₂SO₄ at pH 7.8. When well drained, the enzyme was scraped from the paper and stored in tubes in solid CO₂; the paste retained activity for at least 12 months. For assays, fresh dilutions were prepared in 0.2 M-glycylglycine buffer at pH 7.6 and kept at 0° for not more than 12 hr. This preparation contained the following enzymes: phosphoglucomutase, phosphohexose isomerase, fructose 6-phosphokinase, aldolase, phosphotriose isomerase, glycerol phosphate dehydrogenase, adenylate kinase, and nucleoside diphosphate kinase. At the dilutions used in enzymic assays (see Methods and Results section), the material had little absorption at 340 m μ and did not oxidize reduced DPN.

Yeast alcohol dehydrogenase. The crystalline enzyme was obtained from Sigma Chemical Co.

Intestinal alkaline phosphatase. This was prepared from calf mucosa and purified as far as step 6 in the procedure as described by Morton (1954). Material which had been stored for some years was dissolved in 0.05 M-glycine, pH 9.5 and kept overnight at 2°. Insoluble material was removed by centrifuging.

METHODS AND RESULTS

Paper for chromatography and electrophoresis. Whatman no. 3 paper was washed as described by Connell, Dixon & Hanes (1955) except that treatment with calcium acetate was omitted. This was generously provided by Dr K. Rowan.

Spectrophotometry. Extinctions were measured in an Optica CF4 grating spectrophotometer calibrated for extinction with filters supplied by the National Standards Laboratories, Sydney. Wavelengths were calibrated against known lines of the mercury emission spectrum.

Electrophoretic determination of nucleotides. The following procedures were carried out in a cold room

at about 3°. Samples of 20 or 25 μ l. were applied as bands extending to within 2 mm. of the edges of strips (25 mm. \times 300 mm.) of washed paper. The papers were dipped into 0.04 M-sodium citrate, pH 4.4-4.7, to within 2 cm. of the sample zone. Excess of buffer was removed by blotting with filter paper, and the strips were hung vertically until the wet zones had joined by capillary movement. Samples were usually analysed in quadruplicate and two blank strips were prepared.

The strips were placed on the supporting frame in a horizontal electrophoresis assembly (Evans Electroelenium Ltd.) containing the same citrate buffer. Accurate levelling of papers and buffers was essential. A potential of about 400 v was applied (approx. 14 v/cm.) for 3½ hr. The papers were then removed and dried at 25° in a horizontal position. Nucleotides were located by printing on reflex document paper with light from an unfiltered low-pressure mercury lamp (Philips TUV, 15 w; maximum emission at 254 m μ). The wet prints (after developing and rinsing) were placed on a light-box, covered with clean polythene film, and matched against the electrophoresis paper strips. Nucleotide bands were marked with a 5 mm. margin beyond the apparent limit of u.v. absorption. These zones were cut out, cut into narrow strips and placed in cylindrical funnels (approx. 20 mm. \times 80 mm.) with glass-wool plugs, arranged to drain into 5 ml. volumetric flasks. In subsequent experiments, glass wool was replaced by a 5 mm. bed of no. 12 Ballotini beads. Each nucleotide band was eluted with 5 \times 1 ml. portions of aq. 0.05 N-NH₃ soln. during 30 min. and the combined eluate was made up to 5 ml. Zones from corresponding regions of the blank strips were similarly eluted. Extinctions of blanks and nucleotide eluates were measured at 259 and 290 m μ in 4 cm. cells against the eluting solvent. The true extinction at 259 m μ due to nucleotide was determined from the expression:

$$E_{259 \text{ m}\mu} (\text{sample}) - E_{290 \text{ m}\mu} (\text{sample}) \times E_{259 \text{ m}\mu} (\text{blank}) / E_{290 \text{ m}\mu} (\text{blank})$$

Nucleotide concentrations were calculated from the equation $\epsilon_{259 \text{ m}\mu} - \epsilon_{290 \text{ m}\mu} = 15.4 \times 10^3$ (Bock, Ling, Morell & Lipton, 1956).

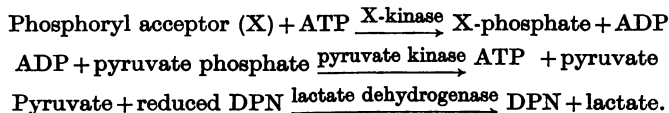
Enzymic determination of nucleotides

Reactive phosphoryl groups (adenosine di- and triphosphate). The oxidation of reduced DPN (decrease in $\epsilon_{340 \text{ m}\mu} = 6.2 \times 10^3$; Horecker & Kornberg, 1948) was followed spectrophotometrically (see Slater, 1953) in the presence of excess of glucose 6-phosphate. Usually the blank, assay and control cells (1 cm.) contained glucose 6-phosphate (2 μ moles), MgCl₂ (2.5 μ moles), glycylglycine-NaOH buffer, pH 7.6 (62 μ moles), and 0.2 ml. of 20% (v/v) solution of muscle-enzyme paste (see Materials section) in the same glycylglycine buffer. The assay and

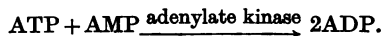
control cells contained enough reduced coenzyme to give an extinction of about 0.8 and the blank and test contained the unknown ($\text{ADP} + 2\text{ATP} < 0.1 \mu\text{mole}$) in a final volume of 3 ml.

Attempts to determine ATP in the presence of ADP (Slater, 1953; procedure C) with commercially available hexokinase preparations gave low values, and ATP was determined by difference between ($\text{ADP} + 2\text{ATP}$) (see above) and (ADP) from the phosphoryl-acceptor method below. In a series of six solutions containing 1–3 μmoles of each nucleotide/ml., the sum of ($\text{ADP} + 2\text{ATP}$) was measured by both the electrophoretic and enzymic methods. The ratio of the results by the two methods was 1.00 ± 0.03 .

Reactive phosphoryl acceptor compounds (adenosine di- and mono-phosphate). These were determined by spectrophotometric measurement of oxidation of reduced DPN, catalysed by muscle lactate dehydrogenase in the presence of pyruvate liberated from phosphoenolpyruvate by phosphoryl transfer (see Strominger, 1955):



Blank and assay cells (1 cm.) contained glycylglycine-NaOH, pH 7.6 (250 μmoles), MgCl_2 (25 μmoles), muscle lactate dehydrogenase containing pyruvate kinase (0.02 ml. of a 5% solution in 0.02 M-glycylglycine buffer, pH 7.6), phosphoenolpyruvate (about 1.2 μmoles) and enough reduced DPN in the assay cell to give an extinction of about 0.8. The total volume was 3 ml. As shown in Fig. 1, there was no oxidation of reduced DPN before addition of phosphoryl acceptor. Equal volumes (usually 0.05 ml.) of a sample containing phosphoryl acceptor ($\text{ADP} + 2\text{AMP} < 0.3 \mu\text{mole}$) were added to each cell. The initial decrease in extinction corresponding to phosphorylation of ADP was complete within 5 min. (Fig. 1). On addition of adenylate kinase (0.02 ml. of a 5% solution; see Materials section) there was a further decrease in extinction (Fig. 1) corresponding to oxidation of 2 equiv. of reduced DPN for 1 equiv. of AMP:



Some samples of lactate dehydrogenase-pyruvate kinase contained traces of adenylate kinase which prevented the accurate determination of ADP in a mixture containing AMP. It was found that lactate dehydrogenase diluted in the phosphoenolpyruvate- MgCl_2 -glycylglycine solution (see above) lost the adenylate kinase activity on storage at 0° for about 14 hr.

The amounts of ADP and AMP were calculated from the change in $E_{340\text{m}\mu}$ (see Fig. 1). In a series of

seven determinations, the ratio of values determined enzymically and electrophoretically for ADP was 1.00 ± 0.03 and for AMP was 0.99 ± 0.06 .

Where suitable kinase preparations are available the enzymic procedure as described here is useful for determination of other phosphoryl acceptors. If the reaction mixture does not contain ADP or ATP, about 1 μmole of the latter should be added to initiate phosphorylation of the acceptor ($< 0.3 \mu\text{mole}$ in this system).

After ADP and AMP had been determined as described above, addition of inosine 5'-phosphate (2 μmoles) to the assay cell caused no further decrease in extinction. Subsequent addition of muscle-enzyme preparation (0.025 ml. of a 20% solution of paste in 0.2 M-glycylglycine buffer, pH 7.6), which contained nucleoside diphosphate kinase, resulted in a rapid oxidation of reduced DPN, corresponding to the phosphorylation of inosine 5'-phosphate. Thus the measured AMP concentration did not include the inosine 5'-phosphate, which could be separately estimated.

Estimation of galactose

Galactose was estimated colorimetrically by the arsenomolybdate method (see Nelson, 1944). In earlier experiments, protein and phosphates were precipitated with $\text{ZnSO}_4\text{-Ba(OH)}_2$ reagent. However, interfering compounds in the galactokinase equilibrium mixtures were not entirely removed by this procedure. In later work, samples of equilibrium mixture (0.3 ml.) which had been freed of protein by the trichloroacetic acid-ether procedure (see below) were applied to 40 mm. \times 300 mm. strips of washed filter paper, and electrophoresis was carried out at 12 v/cm. for 8 hr. in 0.04 M- Na_2HPO_4 at 3°. As an internal standard, 0.025 ml. of galactose (1.00 mg./ml.) was applied to two of the four test strips and to one of the two blank strips.

The compounds which absorbed at 254 $\text{m}\mu$ were first located photographically as already described, and then galactose was located by treatment of marker strips with alkaline silver reagent. In this system the nucleotides and galactose 1-phosphate were well separated from the uncharged galactose which was then eluted as described for nucleotide estimation with five 1 ml. portions of water (final vol., 5.0 ml.).

Duplicate 2 ml. portions of the six eluates were mixed with 0.25 ml. of galactose (100 $\mu\text{g./ml.}$) to bring the galactose concentration to the most suitable region of the standard line. Alkaline copper reagent A + B (2 ml., see Nelson, 1944) was added

to each tube and to a series of standards. After thorough mixing of the contents, the tube was kept for 35 min. in boiling water and cooled for 5 min. under tap water. The contents were mixed with 2 ml. of arsenomolybdic acid reagent, and after 20 min. diluted with water to 20 ml. Extinctions (2 cm. light path) were read at 600 $m\mu$ within 2 min. of mixing. The times of initial mixing and heating were arranged so that each tube was treated in the same way. Extinction was a linear function of the amount of galactose over the range 0–90 $\mu\text{g./tube}$ with an extinction range 0.1–1.0. The mean recovery of the internal standard, added before electrophoresis, was 95 %.

Other procedures

Measurement of pH. This was done at 25.0° with a glass electrode and pH meter which had been calibrated against standard phthalate and borate buffers. Small glass and calomel electrodes for use in narrow tubes were constructed by N. L. Jones, Melbourne.

Removal of protein from equilibrium mixtures. Two procedures were used. Formic acid was added to a final concn. of 10 % (v/v), protein was removed by centrifuging, and a definite volume of the supernatant was freeze-dried in a tube previously marked at the original meniscus. The dry residue was dissolved in water and made up to the initial volume. Alternatively, 1 ml. of trichloroacetic acid (50 % v/v) was added to the equilibrium mixture (8 ml.) in a tube previously marked at the meniscus. After 2 min. at 25°, the solution was cooled to 0° and then extracted with diethyl ether (4×10 ml.). The aqueous solution was concentrated to about 7 ml. at 30° in a stream of N_2 and diluted to 8 ml. with water. Solutions were kept in closed tubes in solid CO_2 until analysed.

No significant difference was found between nucleotide analyses of materials freed of protein by the formic acid and trichloroacetic acid procedures. The latter procedure was used in obtaining the equilibrium concentrations reported here.

Establishment of equilibria

(a) **Initial reactants, adenosine triphosphate and galactose.** Into a tube with a mark at 8 ml. were added galactose (18.5 mg., 102 μmoles), ATP (44 mg., 65 μmoles), MgCl_2 (0.25 M, 0.8 ml.) and water (3 ml.). The solution was stirred with a stream of N_2 and kept at 25.0°, and the pH was brought to 7.0 by addition of 0.25 N-NaOH from a microsyringe. Then 0.075 ml. of galactokinase and 0.01 ml. of adenylate kinase were added. The pH was kept at 7.0 by addition of alkali to neutralize acid liberated on phosphorylation of the galactose, according to the reaction:

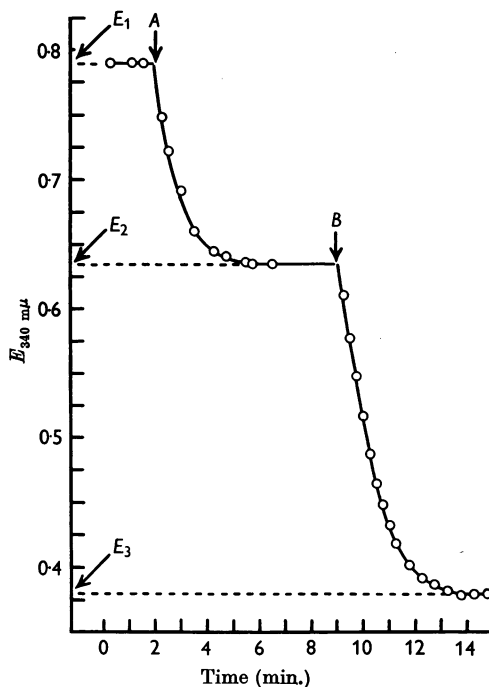
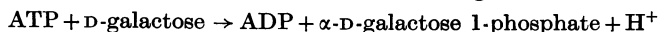


Fig. 1. Determination of ADP and AMP with coupled dehydrogenase-pyruvate kinase-adenylate kinase system. The details of the procedure are given in the Methods and Results section. At A, 0.05 ml. of solution containing ADP (0.065 μmole) and AMP (0.053 μmole) was added. At B, adenylate kinase (0.02 ml.) was added. Extinctions are corrected for dilution to the final volume, 2.6 ml. The concentration of ADP (mM in cell) was calculated from $(E_2 - E_1)/6.2$, and the concentration of AMP (mM in cell) from $(E_2 - E_3)/(6.2 \times 2)$.

Three successive 0.05 ml. portions of galactokinase were then added at approx. 2 hr. intervals and, when no further pH change could be detected, 0.01 ml. of adenylate kinase was added, and the solution was diluted with water to 8.0 ml. After a further 20 min at pH 7.00 and 25°, the solution was freed of protein with trichloroacetic acid (see above). Galactose, ATP, ADP and AMP were estimated as already described. Some inosine 5'-phosphate, adenosine and inosine were formed as a result of adenylate deaminase and phosphatase activities. These compounds were well separated from the adenylyl nucleotides by electrophoresis and caused no interference with enzymic assays. Galactose 1-phosphate was estimated by difference between the initial and final concentrations of galactose. In one experiment (see Table 2) the solution was treated with phosphatase, which liberated all the galactose initially present. There was no conversion of galactose into compounds other than $\alpha\text{-D-galactose}$

1-phosphate as demonstrated by enzymic assays for glucose phosphates (Slater, 1953) and by characterization of the phosphorylated sugar as α -D-galactose 1-phosphate (see Materials section). Results are given in Tables 1 and 2.

(b) *Initial reactants, adenosine diphosphate and galactose 1-phosphate.* Into a tube were put ADP (36 mg., 68 μ moles), α -D-galactose 1-phosphate (51.3 μ moles in 5 ml.), and $MgCl_2$ (0.25 M, 0.8 ml.). As previously described, the pH was brought to 7.0 and after addition of galactokinase and adenylate kinase was kept at 7.0 by addition of 0.25 N-HCl and 0.25 N-NaOH. When there was no further change of pH, water was added to 8 ml. and after a further 20 min. at pH 7.0 the solution was freed of protein with trichloroacetic acid and analysed as described for (a) above. Results are given in Table 1.

DISCUSSION

The free-energy change of phosphoryl transfer from ATP to the α -anomer of D-galactose is so small at pH 7.0 that all the reactants at equilibrium may be measured by specific methods. With the precautions already described, spectrophotometry after electrophoretic purification has proved a satisfactory method for the analysis of AMP, ADP and ATP. The standard error in determination of ADP and ATP by this method was 1-3% of the mean value for 1-5 mM-solutions; a similar precision was possible in the determination of AMP, but longer runs were needed for purification of this compound, and in Table 1 equilibrium constants were calculated from AMP concentrations determined by the enzymic procedure.

Table 1. *Equilibrium constant of the galactokinase reaction*

For experimental conditions see text; the concentration of Mg^{2+} ions was 25 mM.

	Equilibrium concentrations (mM)	
	From ($\alpha + \beta$)-D-galactose and ATP	From α -D-galactose 1-phosphate and ADP
ATP (by electrophoresis)*	1.06 \pm 0.02 (4)	1.51 \pm 0.04 (4)
ADP (by electrophoresis)*	2.78 \pm 0.03 (4)	2.49 \pm 0.01 (4)
ADP (by enzymic assay)	2.81 \pm 0.08 (4)	2.47 \pm 0.02 (3)
2ATP + ADP (by enzymic assay)	4.75 \pm 0.06 (4)	5.30 \pm 0.07 (4)
2ATP + ADP (by electrophoresis)	4.90 \pm 0.04 (4)	5.51 \pm 0.08 (4)
AMP (by electrophoresis)	2.18 \pm 0.12 (4)	1.12 \pm 0.07 (4)
AMP (by enzymic assay)*	2.05 \pm 0.03 (4)	1.17 \pm 0.02 (3)
D-Galactose	1.2 \pm 0.1 (8)	0.37 \pm 0.04 (8)
α -D-Galactose 1-phosphate (by difference; see text)	11.6 \pm 0.1	6.1 \pm 0.1
$\dagger K_{Gal}$	25 \pm 3	27 \pm 3
$\dagger \Delta G'$ (kcal./mole)	-1.9 \pm 0.1	-1.9 \pm 0.1
$[ADP]^2/[AMP][ATP]$	3.6	3.5

* Value used in calculation of equilibrium constants.

$$\dagger K_{Gal} = \frac{[ADP][\alpha\text{-D-galactose 1-phosphate}]}{[ATP][(\alpha + \beta)\text{-D-galactose}]}$$

$$\dagger \Delta G' = -RT \ln K_{Gal}$$

Table 2. *Preliminary galactokinase equilibria*

These results were obtained with 10 mM- Mg^{2+} at pH 7.0 and 25.0°; for experimental details, see text.

Equilibrium concentrations (mM)							
ATP	ADP	AMP	Galactose	Galactose 1-phosphate	$K_{Gal}\dagger$	$\Delta G'$ (kcal./mole) \dagger	$\frac{[ADP]^2}{[ATP][AMP]}$
0.94	2.35	2.83	1.79	11.0	15	-1.6	2.1
0.91	2.40	2.91	1.67	11.1	18	-1.7	2.2
1.11	2.39	2.43	0.82	10.4	27	-1.9	2.1
1.51	2.45	1.83	0.40	9.2	37	-2.1	2.2
1.65	2.50	1.97	0.39	9.2	36	-2.1	1.9
3.3	3.5	2.1	0.47*	7.6	17	-1.7	1.8
3.92	2.36	0.66	0.11	3.9	21	-1.8	2.2
Mean					24 \pm 9	-1.9 \pm 0.2	2.1 \pm 0.2

* After hydrolysis with phosphatase 8.4 \pm 0.8 (for details see text).

\dagger See Table 1.

Phosphoryl transfer from phosphoenolpyruvate to ADP and enzymic reduction of the pyruvate liberated afforded confirmation of the electrophoretic assay of the diphosphate; concentrations measured by the two methods did not differ by more than 1% (Table 1). Extension of this assay by coupling with adenylate kinase (see Methods and Results section and Fig. 1) provided a sensitive method for estimation of AMP; the standard error was about 2% of the mean value. This assay of phosphoryl acceptors is widely applicable if suitable kinases, free of adenosine triphosphatase and other phosphatases, are available. Slater's (1953) enzymic assay of phosphoryl donors ($\sim P$) was used for comparison with the electrophoretic determination of (ADP + 2ATP); differences between the two types of results were not more than 4%, and in the preliminary experiments (Table 2) were less than 3%.

Estimation of galactose was the greatest source of error in these experiments. In Table 1, where results shown were obtained after electrophoretic purification, the recovery of 95% of internal standards, essentially equal in concentration to the unknown galactose, and the standard error (about 10% of the mean) indicate the probable reliability of the analysis. Although the glucose content of the original galactose was less than 1% (for details see Methods and Results section), this would have been a serious contaminant in the small proportion of residual galactose, but for the presence of hexokinase, which catalyses almost complete phosphorylation of glucose in these conditions (Vladimirov *et al.* 1957a; Robbins & Boyer, 1957).

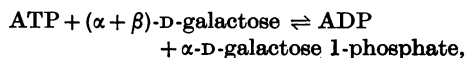
After it had been shown (Table 2) that phosphatase hydrolysed all the esterified galactose to a reducing sugar, α -D-galactose 1-phosphate was estimated as the difference between the initial and final galactose concentrations. Examination of equilibrium mixtures by enzymic procedures (Slater, 1953) and of the hexose phosphate before and after hydrolysis (see Methods and Results section) gave no evidence of conversion of galactose into any compound other than α -D-galactose 1-phosphate. As the ester was the major component at equilibrium its subsequent estimation by difference introduced a relatively small error.

Although the hexose analyses in the preliminary experiments with 10 mM-MgCl₂ (Table 2) were less satisfactory than in later experiments (Table 1), the standard error of individual values of K_{gal} in Table 2 is probably less than 20%. The variation observed in the series of experiments shown in Table 2 (K_{gal} , 24 ± 9) is probably due to competition for Mg²⁺ ions between the phosphorylated reactants resulting in different extents of magnesium complex formation. At the higher magnesium chloride concentration (25 mM) used in the later

experiments (Table 1) there is a considerable excess of Mg²⁺ ions with respect to phosphorylated reactants (cf. Robbins & Boyer, 1957; Burton, 1959) and these differences are apparently avoided. Thus in these conditions K_{gal} was 25 ± 3 with galactose and ATP as the initial reactants, and 27 ± 3 with α -D-galactose 1-phosphate and ADP, despite considerable differences in the proportions of reactants. The effect of the concentration of Mg²⁺ ions on the equilibrium constant of the adenylate kinase reaction is also evident from Tables 1 and 2.

SUMMARY

1. Electrophoretic and enzymic methods are described for specific estimation of compounds present in the equilibria:



and



which are catalysed by galactokinase and adenylate kinase respectively.

2. A general spectrophotometric estimation of the substrates of kinases is described.

3. The equilibrium constant of the galactokinase reaction $[\text{ADP}][\alpha\text{-D-galactose 1-phosphate}]/[\text{ATP}][(\alpha + \beta)\text{-D-galactose}]$ was 26 at pH 7.00 and 25° in the presence of 25 mM-Mg²⁺.

4. The equilibrium constant of the adenylate kinase reaction $[\text{ADP}]^2/[\text{AMP}][\text{ATP}]$ at pH 7.00 and 25° was 2.1 with 10 mM-Mg²⁺ and 3.6 with 25 mM-Mg²⁺.

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Haemolysins in Venoms of Australian Snakes

OBSERVATIONS ON THE HAEMOLYSINS OF THE VENOMS OF SOME AUSTRALIAN SNAKES AND THE SEPARATION OF PHOSPHOLIPASE A FROM THE VENOM OF *PSEUDECHIS PORPHYRIACUS*

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A supply of phospholipase A which was free from other enzymes was required during a study of the antagonism between staphylococcal toxin and certain venoms (North & Doery, 1958). Phospholipase A had already been separated from venoms of the honey-bee (Habermann & Neumann, 1957) and *Crotalus terrificus terrificus* (Habermann, 1957). However, for our purpose venoms of Australian species of snakes were considered as a source of this enzyme. The haemolytic activities of a number of these venoms were first studied by Kellaway & Williams (1933), Holden (1934, 1935), Feldberg & Kellaway (1937, 1938) and Trethewie (1939). Holden (1934) showed that washed red cells of the rabbit were haemolysed by solutions of these venoms at 37°. He observed an increase of activity on the addition of lecithin. It was generally agreed at that time, on chemical and pharmacological evidence, that the haemolytic activities of the venoms of the Australian snakes studied were dependent on the formation of lysolecithin by phospholipase A present in the venoms (Feldberg & Kellaway, 1938; Trethewie, 1939).

It is known that there are two types of venom haemolysins, 'direct' and 'indirect' (Holden, 1935; Neumann & Habermann, 1952; Habermann, 1954). The direct haemolysin lyses washed red cells of certain species readily without added lecithin, whereas the indirect haemolysin lyses such cells only feebly, but haemolysis is markedly enhanced by the addition of lecithin, which is converted into the haemolytic agent, lysolecithin, by the phospholipase A.

Both types of haemolysins have been found among the common venomous species of Australian snakes, the venom of *Pseudechis porphyriacus* being particularly rich in both. This venom was readily available and was chosen as a source of phospholipase A. In this paper the concentrations of both types of haemolysins which have been found in venoms of some Australian snakes will be recorded. Certain other venoms considered to be strongly haemolytic were also included in this study. The separation of phospholipase A from the venom of *P. porphyriacus* will be described and discussed in relation to the occurrence of this enzyme in some other venoms.