The biological significance of these results is not readily apparent. In man, addition of GSH in increasing amounts to either whole blood or haemolysates results in a progressive decrease in the oxygen affinity of the haemoglobin[13], thus producing an effect similar to that produced by 2,3-DPG. Whether this same mechanism exists in sheep is not known. However, since the difference in GSH concentration between GSH^H and GSH^L sheep may be 3- or 4fold, it is possible that the higher levels of 2,3-DPG observed in GSH^L animals are compensating in some way, as yet unknown, for the effect of these low GSH levels, in maintaining the position of the oxygen dissociation curve. The data suggests a negative correlation between GSH and 2,3-DPG and in fact overall $r = -0.44$ ($p < 0.05$). However, within GSH types this relationship is not constant, $r = -0.47$ ($p < 0.05$) within GSH^H and 0.09 (NS) in GSH^L animals.

Tosteson[15] reported an increased activity of sodium-potassium activated adenosine triphosphatase (S-ATPase) in red blood cell membranes from HK sheep. Eaton et al.[16] showed that HK red blood cells had, on average, 31% higher levels of ATP than did LK red blood cells and commented that, with an increased level of S-ATPase activity, HK red cells would be expected to have less ATP than LK cells because of the increased utilization of ATP for the electrolyte pump. Our finding that HK red blood cells have higher levels of yet another important organic phosphate compound raises the possibility that 2,3-DPG might also, directly or indirectly, be associated with ion transport in the sheep red blood cell. This suggestion is strengthened by the observations of Gardos[17] and Parker[18] who have suggested that in man, 2,3-DPG plays a role in potassium transport, possibly by way of 2,3-diphosphoglycerate-phosphatase, the enzyme that breaks down 2,3-DPG. Further investigation into the role of 2,3-DPG in the sheep red blood cell should be of significance.

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A simplified assay for cyclic AMP using protein kinase binding[1]

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Summary. The protein kinase binding assay for cAMP was modified by substitution of adsorption by QAE cellulose for the membrane filtration. This modification obviates the variation of recovery of cAMP with the volume of buffer used to wash the filter. The assay is reproducible and technically simpler than those currently employed.

Introduction. One of the most sensitive methods for the determination of the concentration of adenosine 3′,5′ cyclic monophosphate (cAMP) employs cAMP-dependent protein kinase binding[2–4]. Of all these methods, the method described by Gilman[2] is perhaps the most popular. However, the use of cellulose acetate membrane filters in this method to separate cAMP bound to protein kinase from the free nucleotide presents several problems. Membrane filtration is time consuming, and, more importantly, we found that the recovery of bound cAMP varied with the volume of phosphate buffer used for washing the samples.

Materials and methods. 2 assay systems for the determination of cAMP by the protein kinase binding method were employed. The first was identical to that described by Gilman[2]. Typically, different amounts of cAMP were incubated with 4 µg of cAMP dependent protein kinase (Sigma Chemical Co., St. Louis, Mo.); 28 µg of protein kinase inhibitor (Sigma Chemical Co., St. Louis, Mo.); 2 pmoles of (^{3}H)-cAMP with a specific activity of 27.5 Ci/mmole (New England Nuclear, Boston, Mass.) and 50 mM sodium acetate buffer, pH 4.0 in a total volume of 0.1 ml. The assay mixtures were incubated at 0 °C for 60 min. In the original method[2] millipore filters were used to separate free from bound nucleotide. This step was performed exactly as described, but the volume of sodium phosphate buffer used to wash the membrane was varied. In the present method a 5% (dry wt/vol) QAE cellulose (N,N-diethyl-N-2-hydroxypropylamino cellulose; exchange capacity, 0.55–0.52 meq/g; Biorad, Richmond, Calif.) suspension in water was added to the assay mixture to separate free cAMP from cAMP bound to protein kinase.

Since QAE cellulose is a strongly basic ion-exchanger, it can adsorb various anions including cAMP. However, neither protein kinase nor cAMP bound to protein kinase is adsorbed by the cellulose and this property of the exchanger has been exploited to separate free cAMP from bound cAMP. Before use, the QAE cellulose was soaked in water at least for 24 h at room temperature. The cellulose suspension (2 ml) was thoroughly mixed with the

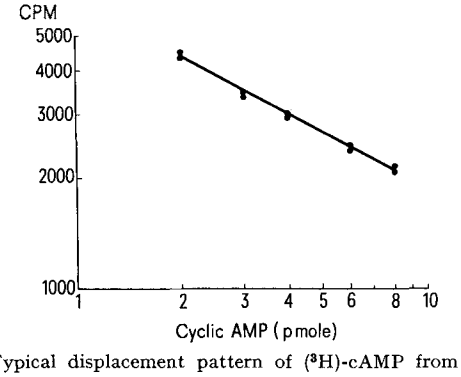

Fig. 1. Typical displacement pattern of (^{3}H)-cAMP from protein kinase by increasing concentration of unlabelled cAMP. Free cAMP was separated from protein kinase bound cAMP by QAE cellulose suspension as described in the text.

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assay mixture and resuspended at intervals for 5 min at 0 °C. Free cAMP adsorbed onto the cellulose was removed by centrifugation at 800 × g for 10 min at 0 °C. One ml of the supernatant was mixed with 10 ml of Aquasol-2 (New England Nuclear, Boston, Mass.) and radioactivity was measured in a Packard Tricarb Scintillation Counter with a counting of efficiency of 45% as determined with an external standard.

Effect of washing the sample with different volumes of phosphate buffer on the retention of protein kinase bound cyclic (^{3}H) AMP by the membrane filter

Vol. of phosphate buffer (ml)	Radioactivity (cpm)
5	12774
10	11621
15	11421
20	10235
25	9108
30	8951
35	8887
40	8230

Cyclic ^{3}H AMP (2 pmoles 27.5 Ci/mmole) was incubated with 4 µg of protein kinase; 28 µg of protein kinase inhibitor in 50 mM sodium acetate buffer, pH 4.0 in a total volume of 0.1 ml at 0 °C for 60 min. After incubation, 1 ml of 20 mM sodium phosphate buffer pH 6.0 was added to the reaction mixture and the mixture was allowed to equilibrate for 5 min at 0 °C. The diluted reaction mixture was then filtered over a Millipore filter (25 mM diameter; catalogue No. HAWP. 025). After washing the sample with different volumes of the buffer, the membrane was dissolved in 1.0 ml of Cellosolve mixed with 10 ml of Aquasol 2 and the radioactivity was measured.

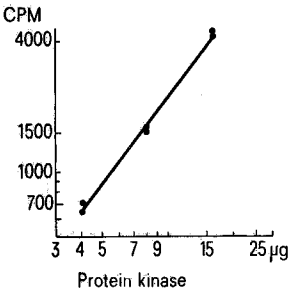

Fig. 2. Effect of protein kinase concentration on the amount of cAMP bound to the enzyme. Varying amounts of protein kinase was incubated in 2 µM labelled cAMP as described in the materials and methods section. Enzyme bound and free cAMP were separated by QAE cellulose suspension.

Results. In the table, the effect of varying the volume of phosphate buffer on the bound cAMP is tabulated. As can be seen, the labelled cAMP decreases progressively with the volume of the buffer employed. This finding indicates that a considerable error can be introduced by relatively small changes in this part of the procedure.

The results of a typical displacement experiment using the QAE cellulose to remove free cAMP are shown in figure 1. As expected, the measured radioactivity decreased as the unlabelled cAMP concentration increased and showed an excellent correlation on a double logarithmic plot. These results are in good agreement with the data of Gilman[2].

In a further experiment, increasing amounts of protein kinase were incubated with 200 pmoles of (^{3}H)-cAMP (50,000 cpm) in otherwise identical mixture described above. After incubation at 0 °C for 60 min, protein kinase bound cAMP was separated by mixing QAE cellulose suspension with the assay mixture and the radioactivity of the supernatant was determined. Figure 2 shows the relationship between the µg of protein kinase added to the reaction mixture and the amount of tritium labelled cAMP bound to the enzyme. It was noted that the amount of cAMP bound to the protein increases with the concentration of protein kinase. At a cAMP concentration of 2 µM, 1.18, 1.98 and 2.81 pmoles of cAMP would bind to 1 µg of protein in the presence of 4, 8 and 16 µg of the enzyme in the assay mixture.

Discussion. The unexpected finding that the volume of washing buffer affects an assay for cAMP led to a quest for a more reproducible and technically simpler method. Separation of protein bound cAMP from the free nucleotide by QAE cellulose suspension is quantitative, and more easily performed than by membrane filtration and, unlike the membrane filtration technique, the QAE cellulose adsorption method does not need any special apparatus. Like QAE cellulose, activated charcoal coated with bovine serum albumin or dextran has also been employed to separate free cAMP from protein kinase bound moiety[3]. However, the extent of adsorption of the free cAMP over cAMP bound to protein kinase by several commercially available activated charcoal varies considerably and the selection of the charcoal has to be carefully made[3]. The variation of recovered counts in replicate samples using the QAE method is minimal. The technique has been successfully utilized for the determinaction of cAMP concentrations in human platelets and lymphocytes.

Serum and liver radioactivity levels in mice after intraperitoneal and subcutaneous injection of [^{14}C]orotic acid[1]

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Summary. [^{14}C]Orotic acid was rapidly distributed in blood after both i.p. and s.c. injection but was not completely absorbed from the peritoneal cavity until 20 min after injection. S.c. injection should be an acceptable alternative to i.p. injection although the incorporation into the liver acid soluble- and RNA-fractions was somewhat delayed after the s.c. injection.

In a previous study, i.p. administration was shown to be as effective as i.v. administration as regards incorporation of [^{14}C]orotic acid into total acid soluble and RNA-fractions in various mouse organs[2]. A problem arising during the analysis of radioactivity levels in metabolites after administration of radionuclides, is the possible interference by unabsorbed radionuclide at the site of injection[2,3]. An i.p. injection may result in a contamination of the liver and other peritoneal organs and i.p. sampled blood is likely to be contaminated to a certain extent by unabsorbed nuclide. In order to ascertain whether these problems could be overcome by using a