inhibitory; 50 mM Mg^{2+} reduced activity in the presence of 2.5 mM Ca^{2+} to approximately 33%. 100 mM Na^+ and K^+ had no appreciable effect on activity, either alone or in combination.

There is little apyrase activity at pH 5; activity increases up to about pH 7, and remains constant between pH 7 and 9. Higher pH's were not tested.

The saliva contains approximately 250 mg protein per ml. At least 11 protein bands were observed following polyacrylamide gel electrophoresis of extracted saliva (figure 2). Apyrase activity was found in 2 positions on the gel. From a graph of the relative mobility of the protein standards vs log molecular weight, band I has a mol. wt of about 120,000 and band II has one of about 1.2×10^6 daltons. Band I activity occurred in a region containing protein bands but none were present in the region of band II. The pattern of staining and the position of the apyrase activity in the 2 gel regions was found consistently, both with saliva extracted from glands and with the buffer into which insects had salivated. The activity of both regions was calcium dependent, and was not found when heated saliva was used.

An attempt was made to separate the salivary proteins by gel filtration on Sephacryl S-200. There were 4 major peaks of absorption at 280 nm. Apyrase activity was found in 2 well separated peaks. The 1st peak, when electrophoresed, gave a band of apyrase activity in the same region as band I (figure 2). There was insufficient activity in the second peak to examine it further. No peak corresponding to band II (figure 2) was seen. A large peak, separating the two with apyrase activity, contained ferroprotoporphyrin IX, the prosthetic group of haemoglobin. This substance is associated with a pigment in the saliva derived from the haemoglobin of the blood-meal, which is probably the predominant protein of the saliva[9].

This work has clearly demonstrated the existence of at least 2 enzymes in *R. prolixus* saliva with apyrase activity, which are calcium-dependent and heat-labile. The maintained activity over high pH's might be due to 2 enzymes with overlapping pH optima. The salivary apyrase is highly active; for comparison, partly purified potato apyrase has been shown[10] to have an activity at 1 mM ATP of about 60 $\mu moles \cdot min^{-1}\ mg^{-1}$ compared to about 12 $\mu moles \cdot min^{-1}\ mg^{-1}$ for the salivary apyrase which is totally unpurified.

The function of the salivary apyrase(s) can only be speculated upon. *R. prolixus* appears to utilize ATP as a signal chemical during feeding; it should be important to remove such a signal after it had been received to avoid saturation of receptors. Alternatively, ATP might be released from damaged cells during the initial probing of the insect, and consequently may need to be removed to prevent masking of signals from the ATP in blood cells. Perhaps more likely is a role in the prevention of clotting of ingested blood. The blood meal in *R. prolixus* remains fluid for many days after feeding; the saliva has previously been shown to have anticoagulant activity[11]. Platelet aggregation is a component of the early stages of clot formation, and depends on the levels of free ATP and ADP released from activated platelets. The salivary apyrase system could maintain free ATP levels sufficiently low to prevent platelet aggregation. Perhaps relevant is the observation that tsetse flies also appear to possess a salivary apyrase[12] whereas the mosquito *Culiseta inornata* does not; blood remains fluid in tsetse flies after ingestion, but clots in mosquitoes[13]. Such a function might also be important in preventing platelet plugs forming round the mouthparts during feeding.

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2 J.J.B. Smith and W.G. Friend, J. Insect Physiol. *22,* 607 (1976).
3 J.J.B. Smith, J. exp. Biol. *78,* 225 (1979).
4 J.J.B. Smith and W.G. Friend, J. Insect Physiol. *22,* 1049 (1976).
5 E.S. Baginsky, P.P. Foa and B. Zak, Clin. chim. Acta *15,* 155 (1967).
6 M.M. Bradford, Analyt. Biochem. *72,* 248 (1976).
7 G.L. Peterson, Analyt. Biochem. *83,* 346 (1977).
8 H. Adam, in: Methods of Biochemical Analysis, p.573. Ed. H.V. Bergmeyer. Academic Press, New York 1963.
9 V.B. Wigglesworth, Proc. R. Soc. B. *131,* 313 (1943).
10 O. Cori, A. Traverso-Cori, M. Tetas and H. Chaimovich, Biochem. Z. *342,* 345 (1965).
11 K. Hellman and R.I. Hawkins, Nature, Lond. *201,* 1008 (1964).
12 A. Mews, personal communication.
13 Unpublished data.

Phosphorylation of human red cell and liver pyruvate kinase. Differences between liver and erythrocyte L-type subunits[1]

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Summary. Purified PK from human erythrocyte was phosphorylated by cAMP-dependent protein kinase type I from human erythrocyte membrane; this phosphorylation affected only the 'heavy' L′ subunit but not the L subunit. On the other hand, the L subunit of liver PK was highly phosphorylated. Thus it appears that the L subunits from erythrocyte and liver PK are not identical protein molecules.

Red blood cell pyruvate kinase (PK) shares several kinetic and immunologic characteristics with the liver enzyme[2-4] and it has been suggested that liver and erythrocyte enzymes might be generated from a 'common precursor subunit' (L′ type) which would be transformed into the liver type subunit (L type) by partial proteolysis[5,6]. Both 'precursor' and liver type subunits are present in erythrocytes (L'_E and L_E) with a ratio which differs from one preparation to another according to erythrocyte age and the purification method used. Phosphorylation of red cell pyruvate kinase is still open to discussion. Dahlquist-Edberg found that red cell PK in rats could not be phosphorylated by the catalytic subunit of rat liver protein kinase[7]. On the other hand Marie et al.[8] observed endogenous phosphorylation of the two subunit types of human red cell pyruvate kinase after incubation of the red cell with inorganic phosphate ^{32}P.

In this paper we demonstrate differences between the

phosphorylation of erythrocyte and of liver PK[1] subunits by cAMP dependent protein kinase from human erythrocyte membrane.

Material and methods. 1. Preparation of enzymes. Erythrocyte PK was purified from hemolysates by ammonium sulfate precipitation at 40% saturation, phosphocellulose chromatography and affinity chromatography on Sepharose 6B cibacron blue. Degree of enzyme purification was 2500 with a yield of 25%. cAMP-dependent protein kinase was extracted from human erythrocyte membranes at low ionic strength and purified by DEAE-cellulose and ATP-agarose chromatography according to the previously reported method[9]. This protein kinase has been identified as a type I cAMP-dependent histone kinase[9].

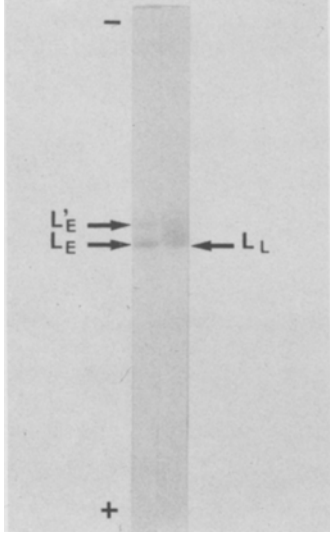
2. Phosphorylation and dephosphorylation of pyruvate kinases. Samples of PK were dissolved in acetate buffer 0.05 M, pH 6.5 + Mg acetate 10 mM + BME 1 mM + glycerol 15% (v/v) and dialyzed against 500 volumes of the same buffer (buffer C). Phosphorylation assays were performed in buffer C in a total volume of 70 µl with the following reagents (final concentration): PK 1 mg/ml, ATP 0.25 mM (γ ^{32}P-ATP), cAMP 5 µM, protein kinase 5–50 µl; incubation time varied from 15 to 60 min according to the assays. For overall phosphorylation measurement, the reaction was stopped by trichloroacetic acid and the assay was completed as previously published[9]; for each assay reaction 1 sample was stopped by 1% SDS for subsequent use in SDS-PAGE. For the liver PK phosphorylated samples were submitted to SDS-PAGE without measurement of overall phosphorylation. In our preliminary experiments, purified erythrocyte PK appeared to be less phosphorylated than liver PK. A possibility existed that erythrocyte PK was in a partially phosphorylated form; with the aim of eliminating such a hypothesis erythrocyte PK was submitted to dephosphorylation by a phosphatase as follows.

200 µg erythrocyte PK was dephosphorylated by 500 µg of alkaline phosphatase from *Escherichia coli* (Sigma). The enzymes were incubated for 4.5 h at 25 °C in Tris HCl buffer 20 mM, pH 8.5 + Mg acetate 5 mM + BME 1 mM + glycerol 15% v/v. After incubation, the enzymes were adjusted to pH 6.5 by dialysis against buffer C and phosphorylated as described above.

3. Kinetics of PK were performed as previously reported[10].

Results and discussion. In our experimental conditions, human erythrocyte PK was phosphorylated by cAMP-dependent protein kinase type I of red cell membrane; this phosphorylation was limited to about 0.3 moles of phosphorus per mole of PK and not modified by enzyme dephosphorylation or exposure to FDP. Liver PK was highly phosphorylated. SDS-PAGE of erythrocyte PK showed 2 types of subunits with slightly different molecular weights. Only the 'heavy' subunit L'_E was phosphorylated. The electrophoretic mobility of liver PK is identical to that of the 'light' subunit L_E of erythrocyte enzyme; radioactivity incorporated in the liver PK band (L_L) of SDS-PAGE was similar to that incorporated in the heavy chain L'_E of erythrocyte enzyme. Thus the protein kinase we used was active only towards 1 of the 2 subunit types of erythrocyte PK. The 'light' subunit seems to have lost the phosphorylatable site(s). This loss, which is probably due to a partial proteolysis, might possibly be an artefact produced in the course of enzyme purification by proteolytic enzymes[8]. However, such proteolysis could also be a physiological process in the red cells leading to a modified L type subunit characterized by the loss of a peptide fragment bearing the phosphorylatable site[7]. L liver subunit (L_L) was phosphorylated at the same level as L' erythrocyte subunit (L'_E). This confirms that L_L and L'_E bear the same phosphorylatable site[8]. However, in spite of an identical electrophoretic mobility, the erythrocyte PK 'light' subunit L_E is not identical to the L subunit of the liver enzyme since it was not phosphorylatable. If it is true that L_E and L_L are derived from a common L' precursor by partial proteolysis, the proteolytic process leading from L' to L_L differs from that producing L_E from L'. Since L_E was not phosphorylatable, only 1 part of the purified erythrocyte PK would bind ^{32}P transferred from γ(^{32}P) ATP: this probably explains why the overall phosphorylation of our erythrocyte PK was lower than that usually reported for liver enzyme (4 moles of phosphorus per mole of PK) and that consequently the kinetic characteristics were not modified. Differences between various results reported can possibly be explained by the very different experimental conditions used by each research team. Dahlquist-Edberg studied the phosphorylation of PK from rat red cells by rat liver protein kinase[7]. Marie et al. performed phosphorylation on intact human red blood cells incubated with ^{32}P inorganic phosphate before PK purification[8]: they used a physiological but complex system in which several protein kinases may be involved.

Conclusion. The L' subunit but not the L subunit of erythrocyte PK is phosphorylated by cAMP-dependent protein kinase from erythrocyte membrane. Since the L subunit of liver PK is phosphorylated, L subunits of liver and erythrocyte PK are not quite identical protein molecules.



SDS-PAGE of pyruvate kinase purified from human red cell (left) and liver (right) (Coomassie blue staining). L'_E 'heavy' subunit of erythrocyte PK. L_E 'light' subunit of erythrocyte PK. L_L liver PK subunit.

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2 J. Marie, A. Kahn and P. Boivin, Biochim. biophys. Acta *438*, 393 (1976).
3 J. Marie, A. Kahn and P. Boivin, Biochim. biophys. Acta *481*, 96 (1977).
4 J.S. Peterson, C.J. Chern, R.N. Harkin and J.A. Black, FEBS Lett. *49*, 43 (1974).
5 J. Marie, H. Garreau and A. Kahn, FEBS Lett. *78*, 91 (1977).
6 A. Kahn, J. Marie, H. Garreau and E.D. Sprengers, Biochim. biophys. Acta *523*, 59 (1978).
7 U. Dahlquist-Edberg, FEBS Lett. *88*, 139 (1978).
8 J. Marie, L. Tichonicky, J.C. Dreyfus and A. Kahn, Biochem. biophys. Res. Commun. *87*, 862 (1979).
9 P. Boivin and C. Galand, Biochem. biophys. Res. Commun. *81*, 473 (1978).
10 P. Boivin, C. Galand and M.C. Demartial, Path. Biol. *20*, 583 (1972).