Assay of *cyclo*-3′,5′-Adenosine Monophosphate (*c*AMP) in Sub-picomole Quantities Using a Covalent Protein-Sepharose Complex[1]

Two types of assay based on the competitive adsorption ('binding') of cold and radioactively labelled *c*AMP to specific 'binding protein' molecules are known. The first is an immunological method employing antibodies produced against *c*AMP[2], the second uses naturally occurring, preformed '*c*AMP binding proteins' extracted from various tissues[3]. We have found both to have their drawbacks: The antigenicity of *c*AMP (free or coupled to a carrier) is low and the preparation of antibodies rather tricky. After the establishment of equilibrium between the amount of *c*AMP 'bound' (c_b) and free in solution (c_f), it is difficult to achieve a clean-cut separation between the two phases so that only either c_b or c_f, but not both, can be measured, thus reducing the precision and introducing a number of experimental problems.

We have therefore covalently attached partially purified 'binding proteins' from cow adrenals to Sepharose 2B by the cyanogen bromide method[4]. The resulting product is very stable and retains its specific binding properties unchanged during months at 1–4 °C. The mean association constant for *c*AMP at pH 7.4 is $K_{ass} \simeq 4.5 \times 10^8$ l/mole, and the maximal binding capacity $P \simeq 1.5$ picomoles/ml of protein-Sepharose suspension (assays in 0.03 *M Tris*-HCl buffer). Equilibration with cold and tracer (tritium) *c*AMP is achieved within less than 1 h at 4 °C without shaking, and the 'Sepharose protein *c*AMP complex' can be rapidly and quantitatively isolated by filtration over a 35 μm polyester net (filtration and washing take about 20 sec.). Both c_f and c_b can be determined by scintillation counting, because neither sepharose nor polyester net interfere. The compound has been used for quick and reliable assays of picomole and subpicomole amounts (down to about 0.2 pmoles) of *c*AMP in biological samples. The results compare very well with those of parallel immunological assays run on less diluted samples with 1–2 pmoles of *c*AMP.

Slight pH and salt concentration effects on K_{ass} and P_o as well as the *Hill*-coefficient were observed. It is therefore necessary to measure both standard and unknown at the same pH and salt concentration (p.e. addition of NaCl to $\simeq 0.7 M$). No specific salt effects are shown. None of the common nucleotides interfere with the assay at less than $\simeq$ 500 times the *c*AMP concentration; only *cyclo*-3′, 5′-inosine monophosphate ($K_{ass} \simeq 1 \times 10^8$ l/mole) could be disturbing.

Assay. 50 μl of standard or unknown *c*AMP solution; 50 μl of ^{3}H-*c*AMP solution (containing $\simeq$ 0.2 pmole of tracer, 15 Ci/mole); 200 μl of 'sepharose coupled *c*AMP binding protein' suspension (containing $\simeq$ 0.3 pmole of binding sites). Keep for 1 h at 4 °C. Pipette an aliquot of supernatant (no need for centrifugation) into counting vial and count ($\rightarrow c_f$). Filter through 35 μm polyester net, rinse and wash with 1.5–2 ml of 50 m*M Tris*-HCl 5 m*M* theophyllin buffer. Transfer net plus precipitate into a scintillation vial and count ($\rightarrow c_b$). Calibration curves were determined anew for every assay series.

Zusammenfassung. Es wird eine neue, einfache und ausserordentlich empfindliche Bestimmungsmethode für Mengen von 0.2–1 Picomol *c*AMP beschrieben. Sie verwendet spezifische Bindungsproteine, die durch covalente Verknüpfung mit Sepharose unlöslich gemacht und stabilisiert wurden, und arbeitet nach dem Isotopen-Kompetitionsverfahren.

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Inhibition of Liver Lactate Dehydrogenase by Serotonin: Possible Relationship to Endotoxin Effects[1]

Though *E. coli* endotoxin does inhibit the function of isolated mitochondria[2,3], it shows no general interference with energy metabolism in vivo, as long as the shock state produced by its i.v. injection has not entered the stage of hemodynamic decompensation[4]. This suggests that primary intracellular effects of endotoxins, if present, are slow to develop, probably because of a size restricted penetration of cellular membranes. However, the in vivo injection of endotoxin releases low-molecular, humoral 'mediators', which might be a potential cause of early intracellular alterations. One of these – epinephrine – activates the adenyl cyclase system[5], but there is no information on a possible interference with enzyme activities by the other mediators. As will be shown in this paper, serotonin is capable of inhibiting liver lactate dehydrogenase (LDH).

Materials and methods. Five adult, outbred healthy rabbits were anesthetized with Nembutal® 30 mg/kg i.v., and the liver was rapidly removed. 10 g of liver tissue were minced and homogenized in cold 0.25*M* sucrose + 5 m*M*

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Inhibition of rabbit liver cytosol LDH by serotonin

Cytosol No.	1	2	3	4	5	$\bar{x} \pm$ SD	95% confidence interval of x
Max. inhibition (% of control)	87.4	87.0	93.2	81.0	81.5	86.0 ± 5.0	81.6– 90.4
ST concentration for max. inhibition (γ/ml)	985	998	985	1011	985	992.8 ± 11.6	982.6–1003.0
C_{50} (γ/ml)	72.8	74.5	79.8	106.4	79.8	82.7 ± 13.6	70.7– 94.6
C_{10} (γ/ml)	10.64	10.64	13.30	4.52	6.65	9.15 ± 3.51	6.07– 12.23

cysteine, pH 7.4. A preliminary centrifugation at 1,500 *g* removed nuclei and non-disrupted cell fragments. The supernatant obtained was then centrifuged at 105,000 *g* for 1 hour. The final supernatant – cytosol according to the terminology of Lardy – contains the soluble glycolytic enzymes. 1 ml aliquots of this preparation were stored at −20 °C and defrosted prior to analysis. Control experiments showed that LDH was stable for at least 4 h following thawing.

LDH was assayed by the method of KORNBERG[6], without any modifications. A 1:100 dilution of cytosol was made up in 0.25 *M* sucrose + 5 m*M* cysteine, pH 7.4. The assay volume was brought to 2 ml with double-distilled water (controls); with a $10^{-2}M$ solution of epinephrine, norepinephrine, histamin or serotonin; with further dilutions of these mediators; or with *E. coli* 0111 endotoxin prepared by one of us (B.U.) and dissolved by shaking for 5–10 min in a mannitol-sucrose solution ($0.225+0.025M$) to a concentration of 6 mg/ml. The following assays – all at pH 7.4 – were run on each cytosol: 1. Control = intrinsic LDH activity. 2. Addition of the $10^{-2}M$ mediator solution instead of water. The LDH activity change in this medium was expressed as percent of the control value and termed 'maximum activity change' (activation or inhibition). 3. The mediator concentration producing a half-maximum activity change was determined ('C_{50}'). 4. We then established the mediator concentration yielding a 10% change of the control activity ('C_{10}'), this being considered to represent a significant difference. 5. Finally, we rechecked the control or intrinsic LDH activity.

Results. Endotoxin, epinephrine, norepinephrine and histamin showed no significant interference with rabbit liver cytosol LDH activity. By contrast, the maximum serotonin (ST) concentration as defined above inhibited LDH almost completely. The mean ST concentration producing a 10% inhibition was 9.15 γ/ml. The complete results are given in the Table.

Discussion. Although one study using labelled ST revealed an increase of arterial blood radioactivity during hemorrhagic shock[7], there are no indications that ST is of major importance in that state[8–10]. By contrast, the massive ST release in the initial stage of endotoxin shock is well documented[8, 9, 11–13]. The average ST concentration of 9.15 γ/ml which produced a 10% inhibition of liver cytosol LDH in the present study is only about twice the physiological ST level of 4–6 γ/ml in rabbit blood[14, 15]. If the same measured oxygen debt is experimentally induced in a hemorrhagic and an endotoxic shock, lactate production is much smaller in the latter than in the former state[14]. In human patients, the blood lactate levels reflecting a 50% mortality rate are similarly lower in endotoxic than in hemorrhagic-traumatic shock[16]. It is tempting to speculate that the inhibition of liver LDH by serotonin recorded here may in some way be related to this discrepancy.

However, there is no lack of points still requiring clarification. Several authors[8, 9, 11–13] report a rapid and persistent fall of blood serotonin levels to 20% and less of control values, following the early explosive release. Apart from possible methodological uncertainties and the question of a brief or protracted ST liberation, we do not know whether such a decrease of blood serotonin levels is due to its rapid breakdown only, or if the ST released penetrates into cells and exerts intracellular effects of the type demonstrated here. At any rate, it appears that the action of serotonin may not be confined to blood vessels, but that it is also potentially capable of interfering directly with metabolic events.

Zusammenfassung. Serotonin hemmt die Lactatdehydrogenase im Lebercytosol von Kaninchen in vitro. Für eine 10%ige Aktivitätshemmung gegenüber den Kontrollwerten genügte eine durchschnittliche Konzentration von 9,15 γ/ml, die nur etwa zweimal höher liegt als der normale Blutspiegel. Dieser Effekt ist möglicherweise an der im Verhältnis zum Sauerstoffdefizit auffallend geringen Lactatbildung im Endotoxinschock beteiligt.

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