by ouabain could be resolved into two phases. But in contrast to our results particularly the contribution of the 'high-affinity' site appeared to be reduced in the rat. Therefore, presumably, the 'two-independent-receptors' hypothesis proved statistically superior over the assumption of negative cooperativity in the analysis of Noel and Godfraind[15]. Evidence in favor of heterogeneous inhibition of Na,K,Mg ATPase by ouabain was also presented by Mansier and Lelievre[16]. Perfusion of rat hearts with Ca-containing or Ca-free solutions before homogenization revealed a differential effect of ouabain on enzyme activity. The findings suggested the existence of either two isozymes, in accordance with previous results on brain particles[17], or alternatively, of one class of enzyme molecules with a Ca-dependent, differential sensitivity to ouabain.

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Immobilization of chicken liver fructose 1,6-bisphosphatase on CNBr-activated Sepharose[1]

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Summary. Chicken liver fructose 1,6-bisphosphatase is readily immobilized on CNBr-activated Sepharose. The immobilization alters some enzymatic properties. They include broader pH activity curve, loss of activation by K^+ or NH_4^+, increased resistance to inactivation by trypsin, decreased sensitivity to AMP inhibition, and loss of cooperative interaction among AMP-binding sites. The immobilized enzyme retains about 38% or 19% of the specific activity of the native enzyme when the activity is measured in the absence or presence of K^+, resepctively.
Key words. Immobilization; chicken liver fructose 1,6-biphosphatase; CNBr-activated Sepharose.

Fructose 1,6-bisphosphatase (Fru-P_2ase, EC 3.1.3.11) was first discovered in rabbit liver by Gomori in 1943[2]. It has since then been shown to play a key role in gluconeogenesis. During the past two decades, Fru-P_2ases from many sources have been extensively investigated. However, research on immobilization of Fru-P_2ase has attracted little attention. In 1973, Falb et al.[3] reported the attachment of 'alkaline' form of rabbit liver Fru-P_2ase to aminoethyl cellulose via glutaraldehyde. This enzyme polymer adduct retained only about 1% of the specific activity of the native enzyme. These researchers gave no information about the properties of this immobilized enzyme except that the pH optimum was shifted from 9.3 to 8.5. In this communication, we report some properties of chicken liver Fru-P_2ase immobilized on CNBr-activated Sepharose.

Materials and methods. CNBr-activated Sepharose 4B and 6MB, trypsin from bovine pancreas, and other chemicals used in this study were all purchased from Sigma Chemical Company. Before use, activated Sepharose (1 g) was swollen in 100 ml of distilled water. It was then washed for 15 min on a sintered glass filter with 300 ml of 1 mM HCl. The gel was washed thoroughly with 0.1 M $NaHCO_3$ solution (pH 7.9) containing 0.5 M NaCl and 0.2 mM EDTA. Fru-P_2ase was purified from chicken liver by the procedure previously described[4]. The concentration of the purified enzyme was determined by the extinction coefficient at 280 nm ($E^{0.1\%}_{1\,cm} = 0.71$) or by the method of Lowry et al.[5] using purified Fru-P_2ase as standards.

The activity of Fru-P_2ase was assayed by measuring the release of P_i according to the method of Tashima and Yoshimura[6]. Unless otherwise indicated, the reaction mixture (1.0 ml) contained 25 mM triethanolamine/25 mM diethanolamine-HCl buffer (pH 7.5), 1.5 mM $MgCl_2$, 1 mM cysteine, 0.1 mM EDTA, 0.05 mM fructose 1,6-bisphosphate (Fru-1,6-P_2), and an appropriate amount of Fru-P_2ase (native or immobilized). The reaction was initiated by the addition of Fru-1,6-P_2 and was carried out with gentle shaking at 25 °C. It was terminated after 5 min by the addition of the color developing agent[6]. After 15 min, the absorbance at 650 nm was determined and compared to values obtained with P_i standards. In monitoring the Fru-P_2ase activity in the supernatant fluid during immobilization reaction (see below), a continuous spectrophotometric assay was used. The reaction mixture was the same as described above except that 0.2 mM NADP and 1 unit each of phosphoglucose isomerase and glucose-6-P dehydrogenase were additionally included. Fru-P_2ase activity was determined by following the rate of formation of NADPH at 340 nm.

Immobilization of Fru-P_2ase on CNBr-activated Sepharose 4B or 6MB was carried out as follows: moist Sepharose (equiv. 1 g dry wt) was added to 5 ml of 0.1 M $NaHCO_3$ solution (pH 7.9) containing 0.5 M NaCl, 0.2 mM EDTA, 4 mM Fru-1,6-P_2, and 2.0 mg of purified enzyme. The reaction mixture was incubated at 25 °C with gentle shaking for 30 min and the fluid was removed by vacuum filtration (filtrate I). To remove the trace of

enzyme that was adsorbed but not covalently bound to Sepharose, the moist gel was washed five times on a sintered glass filter, each with 6 ml of 0.5 M NaCl solution containing 0.2 mM EDTA. The five filtrates were pooled (filtrate II) and to this was added $(NH_4)_2SO_4$ to 75% saturation and Fru-P_2ase was collected by centrifugation (Fru-P_2ase found in filtrate II was less than 1% of that found in filtrate I). In subsequent repeated washings, no Fru-P_2ase activity was detected in the filtrate and the enzyme activity per unit amount of Sepharose remained essentially constant. The amount of enzyme bound to Sepharose was determined by the difference between the initial amount of enzyme added and the amount of enzyme remaining free in supernatant fluid after coupling process, as previously reported[7,8].

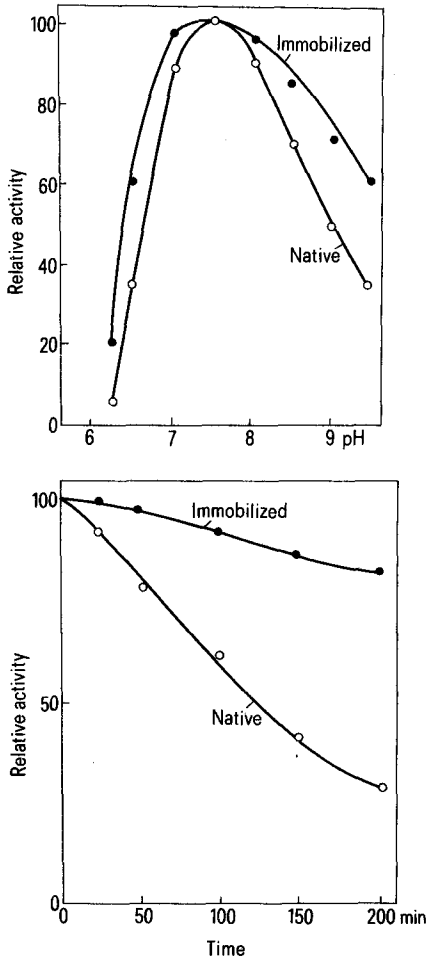

Figure 1. Effect of pH on the activity of native and immobilized Fru-P_2ases. The activity was assayed at the indicated pH values under the conditions as described in 'materials and methods'. The relative activity of 100 corresponds to the sp.act. of 13.2 for the native enzyme or 5.1 for the immobilized enzyme. Each point shown in this figure and figure 2 represents the average values of duplicate assays.

Figure 2. Effect of trypsin on the activity of native and immobilized Fru-P_2ases. To avoid the reaction of trypsin with the remaining active groups in activated Sepharose, the immobilized enzyme used has been pretreated with 1 M ethanolamine (pH 8.0) at 25 °C for 1.5 h. The digestion reaction with trypsin was performed at 25 °C with gentle shaking in 1 ml of 50 mM Tris-HCL buffer (pH 7.5) containing 0.1 mM EDTA, 6 μg trypsin, and (●) 142 mg (equiv. dry wt) immobilized enzyme or (○) 0.25 mg native enzyme plus 142 mg (equiv. dry wt) CNBr-activated Sepharose 4B inactivated with 1 M ethanolamine as described above. At the time intervals indicated, portions were removed and assayed for Fru-P_2ase activity at pH 7.5. The activity of native or immobilized enzyme incubated under the identical conditions but without trypsin remained essentially constant throughout the experimental period.

Results and discussion. In this study, we employed CNBr-activated Sepharose to immobilize Fru-P_2ase. The use of activated Sepharose for enzyme immobilization was first developed by Axen et al.[9] and has since then been used by many to immobilize enzymes. The mechanism for the formation of covalent bonds between the protein and CNBr-activated polysaccharides has been discussed[10].

As summarized in table 1, Fru-P_2ase was readily immobilized by CNBr-activated Sepharose 4B. Within 30 min, about 86% of the enzyme was bound to Sepharose. The immobilized enzyme retained about 38% of the specific activity of the native enzyme. Under the same conditions, activated Sepharose 6MB was less effective than activated Sepharose 4B in coupling this enzyme, but the specific activity of its immobilized enzyme was only slightly lower. Since the enzyme immobilized on Sepharose 4B had higher activity than that immobilized on Sepharose 6MB, it was used in this study to compare with the native enzyme.

The effect of pH on the activities of native and immobilized enzymes is shown in figure 1. Both the native and the immobilized enzymes had the optimum pH at 7.5, but the pH activity curve of the immobilized enzyme became broader. This indicates that immobilization rendered the enzyme less sensitive to pH change.

Like Fru-P_2ases from other sources[11], the enzyme from chicken liver was also activated by K^+ and NH_4^+. This characteristics was, however, abolished after immobilization (table 2). Two possibilities are considered; a) the proper binding of the activating cation to the enzyme was hindered as the result of immobilization; b) the activating cation was incapable of inducing an enzyme conformation more favorable for catalysis when the enzyme was attached to Sepharose. It should be pointed out that in the presence of 0.15 M K^+, the immoblized enzyme retained only about 19% of the specific activity of the native enzyme.

Fru-P_2ase is well-known for its allosteric inhibition by AMP[12]. We have, therefore, investigated the effect of immobilization on the response of the enzyme to this allosteric inhibitor. As the result of immobilization, K_i increased from about 6.3 μM to 40 μM, and Hill coefficient (n) decreased from 2.2 to 1.1. Thus, immobilization resulted in both the decreased sensitivity to

Table 1. Immobilization of Fru-P_2ase on CNBr-activated Sepharose

Sepharose analog	Enzyme (mg)	Sepharose (g dry wt)	Enzyme bound* (%)	Activity of immobilized enzyme Units/g Sepharose	Units/mg protein	% sp. act. of free enzyme**
4B	2.0	1.0	86.2	8.8	5.1	38.6
6MB	2.0	1.0	51.4	4.8	4.7	35.6

* The amount of enzyme bound to Sepharose was determined by the difference between the initial amount of enzyme added and the amount of enzyme remaining free in the supernatant fluid after the binding process. In both cases, the decrease in enzyme activity was exactly proportional to the decrease in the concentration of protein. ** The native free enzyme has the sp. act. (units/mg protein) of 13.2.

Table 2. Effect of K^+ or NH_4^+ on the activity of native and immobilized Fru-P_2ases*

Enzyme	Sp. act. (μmoles/min/mg) Addition None	+ 0.15 M K^+	+ 0.15 M NH_4^+
Native	13.2	25.3	22.4
Immobilized on Sepharose 4B	5.1	4.8	4.7
Immobilized on Sepharose 6MB	4.7	4.6	4.5

* The sources of K^+ and NH_4^+ are KCl and $(NH_4)_2SO_4$, respectively.

AMP inhibition and the loss of cooperative interaction among AMP-binding sites.

The inactivation of Fru-P_2ase by trypsin has been previously reported[13,14]. In this study, we found that the immobilized Fru-P_2ase, as compared with the native enzyme, was much more resistant to inactivation by trypsin (fig. 2). This increased resistance is probably caused by steric hindrance of the approach of trypsin to the critical site of the immobilized Fru-P_2ase.

The native and the immobilized enzymes were inhibited by Zn^{2+} to about the same extent. As previously observed for free Fru-P_2ase[15], the ability of EDTA or other chelators to reverse the Zn^{2+} inhibition of the immobilized enzyme activity decreased greatly if chelators were added to the enzyme after substrate, especially when AMP was also present (data not shown).

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Spontaneous morphine withdrawal from the rat spinal cord[1]

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Summary. A characteristic and reproducible sign of narcotic withdrawal is the naloxone induced increase in arterial pressure. In morphine-dependent rats allowed to undergo spontaneous withdrawal (6–24 h) and then transected at the spinal C-1 level, arterial pressure was maintained at a significantly higher level than either spinal-transected nondependent controls or morphine-dependent, spinal-transected rats pithed from C-1 to L-4. These findings indicate that the morphine-dependent spinal cord, independent of supraspinal influences, is able to exhibit an autonomic component of spontaneous withdrawal.

Key words. Morphine withdrawal; spinal cord; naloxone; blood pressure.

Recent studies in this[4-7] and other laboratories[8] have indicated that the spinal cord plays an important role in the expression of several signs of narcotic abstinence. As an estimate of both the degree of physical dependence and the intensity of withdrawal we have measured the post withdrawal increase in mean arterial pressure (MAP)[9-12] as well as other behavioral signs in the intact, abstinent rat. In morphine-dependent rats transected at the spinal C-1 level, systemic or local intrathecal injection of naloxone elicits a profound and long-lasting increase in MAP which can be abolished by systemic injection of autonomic blocking agents or by spinal pithing[6,7]. The dependent spinal cord, isolated from supraspinal control, should provide a relatively simplistic model for studying the mechanisms of dependence and withdrawal compared with the higher CNS. However, one important criterion for spinal mediated withdrawal has not yet been fulfilled, that is, whether the spinal cord can undergo spontaneous withdrawal. The purpose of this study, therefore, was to determine whether the increase in MAP observed in spinal-transected, morphine-dependent rats following naloxone also could be observed when the animal underwent abrupt morphine discontinuation.

Materials and methods. Male, Wistar rats (280–350 g) were anesthetized with methohexital and an aortic catheter was permanently implanted, and exteriorized to a cannula swivel mounted above the home cage to permit continuous morphine infusion or measurement of arterial pressure[9]. On the following day, morphine sulfate was infused through the catheter at a rate of 0.33 ml/h, to deliver a total dose of 35 mg/kg/day. The concentration was adjusted each morning for the next 4 days to deliver 50, 75, 100 and 100 mg/kg/day, respectively. This schedule was previously determined to induce physical dependence[11,12]. Morphine-dependent or saline-infused control rats (nondependent) were anesthetized with halothane, artificially respired and the spinal cord transected at the C-1 level. Halothane was discontinued and 30 min allowed for recovery prior to any measurements. In some animals the spinal cord was pithed with a 14 gauge trochar from C-1 to L-4.

Results. Dependent unanesthetized, freely-moving rats were deprived of morphine for 6 or 24 h (the time at which withdrawal signs first begin, and the time at which they are maximally expressed, respectively)[9]. MAP was elevated in the abstinent group compared with nondependent controls (table 1), although this was significant only in the 24-h abstinent group. Following spinal transection MAP again was higher in abstinent groups although this was not apparent in dependent, spinal pithed rats. Continuous measurement of blood pressure for 1 h revealed that MAP remained elevated throughout the 60-min observation period, in fact, the 6-h abstinent group began with a slightly lower starting MAP but eventually reached the 24-h dependent level (fig.). Again nondependent controls and dependent, spinal pithed rats maintained a lower MAP.

At the completion of the 1-h measurement period in the abstinent rats MAP remained elevated with respect to the nondependent controls; with respect to abstinent, spinal pithed animals; and, with respect to dependent rats which had not been deprived of morphine prior to C-1 spinal transection (table 2). At this