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Influence of Urea and Organic Solvents on the Activity of Immobilized *myo*-Inositol-1-Phosphate Synthase Containing Active, Self-Regenerating Coenzyme (NAD^+) on the Same Matrix

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Abstract

myo-Inositol-1-phosphate synthase (EC 5.5.1.4.) from rat testes, an NAD^+-containing enzyme that converts D-glucose 6-phosphate to 1L-*myo*-inositol-1-phosphate was immobilized together with its cofactor and bovine serum albumin by crosslinking with glutaraldehyde at pH 4.5. The cofactor is reduced and reoxidized during the reaction cycle, thus forming a self-regenerating system with respect to the cofactor. The behavior of this immobilized enzyme/cofactor system in presence of organic solvents and urea and the activating effect of these compounds on the enzymatic activity were studied and discussed in the paper.

Index Entries: *myo*-Inositol-1-phosphate; NAD; immobilized enzyme; organic solvent, effect on immobilized enzyme activity; urea, effect on immobilized enzyme activity; coenzyme NAD^+, self-regenerating; regenerating, coenzyme NAD^+; synthase, *myo*-inositol-1-phosphate.

Introduction

It is obvious that the physical properties of immobilized enzymes differ from those displayed by the native enzyme in solution. This owes to the influence of the matrix-dependent microenvironment, containing charged groups in fixed positions on the matrix, the resulting change in ion concentration, the possible change in the

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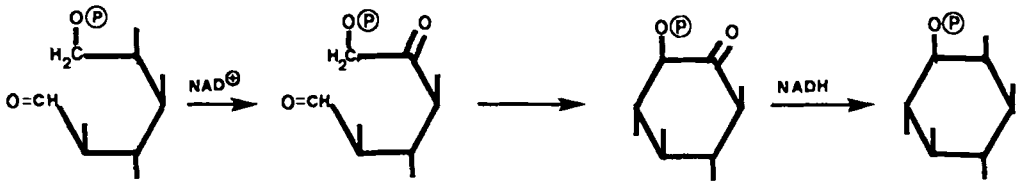

D-Glucose 6-phosphate | D-xylo-Hexos-5-ulose | 2 L-2-Phospho-1,2,5/3,4-pentahydroxycyclohexanone | 1 L-myo-Inositol 1-phosphate

water-structure caused by hydrophobic groups, and the restraining influence of the covalent bonds by which the enzyme is bound to the matrix. These facts should be taken into consideration—not only during the immobilization procedure, in order to obtain the most active immobilized enzyme preparations, but also afterwards—if attempts are made to increase the activity of the obtained gels by treatment with reagents such as organic solvents, urea, and so on. It is shown in this paper that such studies on increasing activity can be useful. The system under investigation was an enzyme immobilized together with its cofactor on the same matrix that was self-regenerating with respect to the cofactor (see scheme 1). Such a system is fully independent of any other influences but those caused by the matrix and the immobilized enzyme itself, and therefore was considered to be most useful.

Materials

All buffer substances, acetic acid, ninhydrin, glutaraldehyde, ethanol, and *N*, *N*-dimethylformamide were obtained from Merck, Darmstadt; bovine serumalbumin, NAD^+, D-glucose 6-phosphate, and dithiothreitol were from Sigma, St. Louis; urea was purchased from Schwarz/Mann, Orangeburg, N.Y.

Methods

Immobilization of *myo*-inositol-1-phosphate synthase together with its cofactor NAD^+ was carried out according to Pittner (*1*). The enzyme, purified as described in previous papers (*2—4*), was dialyzed for several hours against a 1*M* acetate buffer, pH 4.5. Small amounts of denatured protein were removed by centrifugation. Enzyme ($1.6–2.0 \times 10^{-3}$ μkat) and bovine serum albumin (650 mg) were dissolved in 30 mL 1*M* acetate buffer, pH 4.5, containing 0.1 mmol glucose 6-phosphate and 0.15 mmol of NAD^+. The mixture was kept at room temperature for 10 min. Aqueous glutaraldehyde solution (6 mL, 2.5%) was added dropwise under stirring. The mixture was kept without shaking at room temperature for 3 h and then stored overnight at +1°C. The gel formed was homogenized and suspended in a 0.15*M* KCl solution that was 50 m*M* with respect to lysine. Then the gel was washed with the same solution followed by a 0.15*M* KCl solution without lysine until the filtrate gave no reaction with ninhydrin. The gel could be stored in

the same solution in the cold at 4°C or frozen at −15°C for prolonged periods without significant loss of activity.

The native enzyme was assayed according to Barnett et al. (*4*). The activity of the immobilized enzyme was determined by incubation of 100–200 mg of wet gel in 2.5 mL of 50 m*M* Tris-acetate buffer, pH 7.7, that was 2 m*M* with respect to glucose 6-phosphate for 4 h in a well-stoppered tube at 37°C on a thermostated shaker. The reaction was stopped by removal of the gel by centrifugation. Aliquots of the solution containing the reaction product were assayed according to (*4*).

Protein concentrations were determined following a procedure given by Jacobs (*5*).

To determine the activity of the enzyme bound per 100 mg dry protein, wet gel (100–200 mg) was assayed for activity as described above. Then the wet gel was dehydrated by lyophylization and the dry weight determined on a microbalance. Dry polyprotein (75 mg) could be obtained from 1 g wet gel.

Results

myo-Inositol-1-phosphate synthase immobilized together with its cofactor NAD^+ by aggregation with bovine serum albumin and the crosslinking reagent glutaraldehyde (see methods section) was assayed for activity according to the method mentioned above. The enzyme bound to the gel had an activity of 1.4×10^{-5} μkat per 100 mg of protein.

Incubation in the Presence of Dimethylformamide, Ethanol, or Urea

Aliquots of the wet gel (200 mg) in 2.5 mL 50 m*M* Tris-acetate buffer, pH 7.7, that was 2 m*M* with respect to the substrate glucose 6-phosphate, containing increasing amounts of dimethylformamide, ethanol (0–100%) or urea (0–8*M*) respectively, were incubated on a thermostated shaker for 4 h at 37°C. The reaction was stopped by removing the gel via centrifugation, and aliquots of the solution containing the product were treated according to the assay procedure mentioned above. The results are listed in Table 1 and shown in Figs. 1–3.

Incubation in presence of dimethylformamide resulted in an increase of activity at any concentration of the solvent, a maximum being situated at a concentration of 40% dimethylformamide, where the immobilized enzyme showed 3.7-fold activity compared to that of the gel without dimethylformamide. However, in absolute dimethylformamide the activity was completely zero. An interesting effect is the appearance of a second maximum of activity in 90% dimethylformamide. An explanation will be given below.

When the gel was incubated in the presence of increasing amounts of ethanol, quite different results were obtained (Table 1, Fig. 2). In the range of 0–60% ethanol, no significant change in activity could be observed, though at higher concentrations the activity decreased drastically, but never, not even in pure ethanol, reached zero.

When urea was used (Table 1, Fig. 3) concentrations up to 2*M* did not change

TABLE 1
Activity of Immobilized NAD^+/*myo*-Inositol-1-phosphate Synthase in the Presence of Increasing Amounts of Dimethylformamide, Ethanol, and Urea

DMF, %	Activity/100 mg wet gel, μkat × 10^{-5}	Ethanol, %	Activity/100 mg wet gel, μkat × 10^{-5}	Urea, *M*	Activity/100 mg wet gel, μkat × 10^{-5}
5	2.51	5	1.76	1	1.33
10	3.09	10	1.18	2	1.62
20	3.39	20	1.62	3	2.21
30	5.01	30	1.76	4	1.62
40	5.15	40	1.48	5	0.29
50	3.98	50	1.53	6	0.74
60	4.27	60	1.50	7	0.59
70	3.25	70	1.08	8	0
80	3.09	80	0.45		
90	4.72	90	0.32		
100	0	100	0.29		

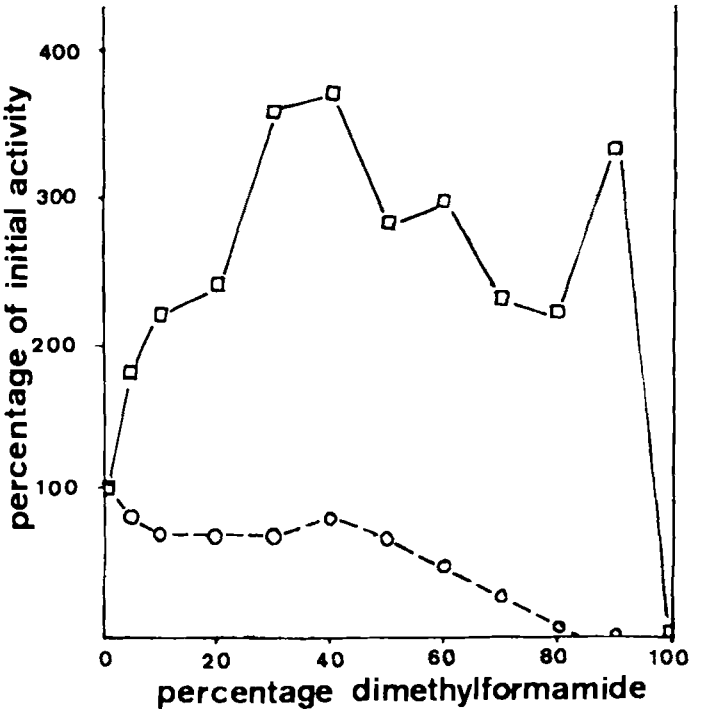

Fig. 1. □—□, Activity of immobilized and native *myo*-inositol-1-phosphate synthase in presence of dimethylformamide. ○–○, Native enzyme under the same conditions.

the activity very much, at 3*M* urea a maximum of activity was observed. After this maximum, the activity gradually decreased, reaching zero at 8*M* urea.

When soluble NAD^+ was added to these series of incubation mixtures, no significant increase of activity could be observed, indicating that the amount of the immobilized cofactor was sufficient for maximal activity.

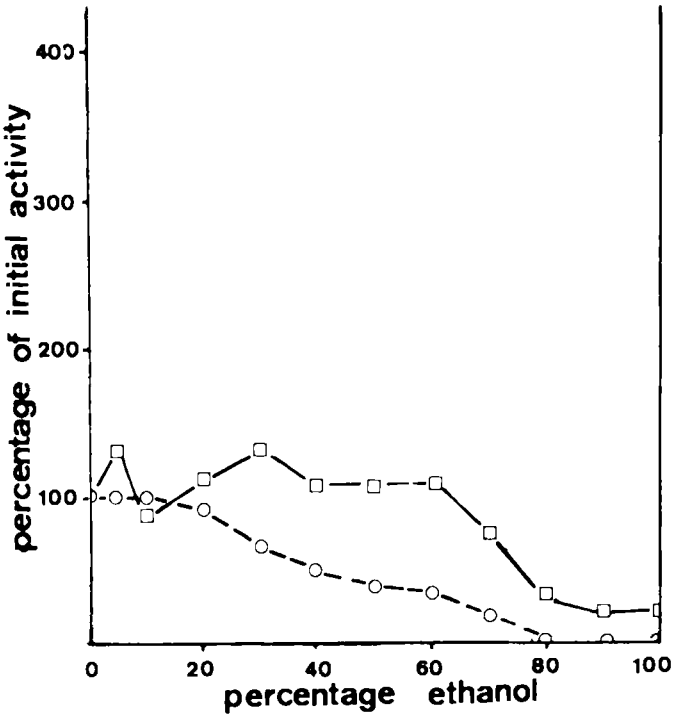

Fig. 2. □—□, Activity of immobilized and native *myo*-inositol-1-phosphate synthase in presence of ethanol. ○—○, Native enzyme under the same conditions.

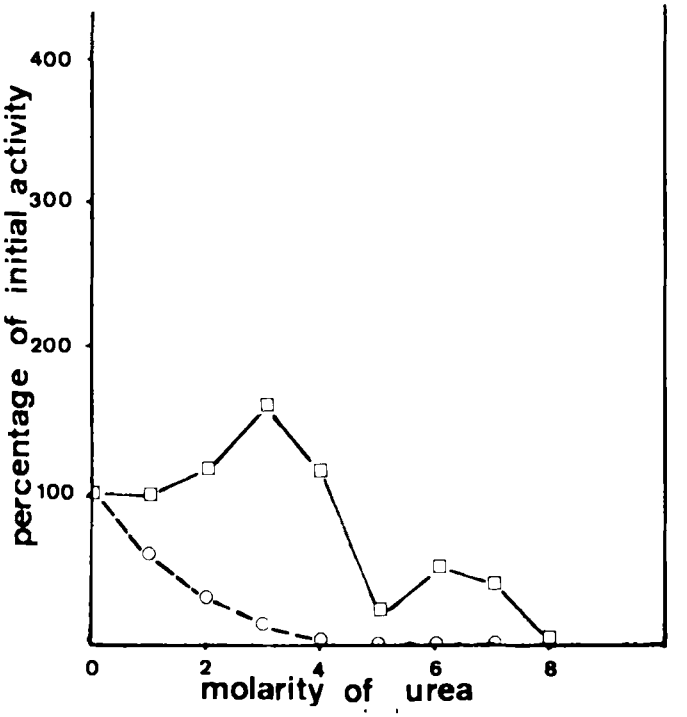

Fig. 3. □—□, Activity of immobilized and native *myo*-inositol-1-phosphate synthase in presence of urea. ○—○, Native enzyme under the same conditions.

TABLE 2
Activity of Immobilized NAD^+/*myo*-Inositol-1-phosphate Synthase after Treatment with Increasing Amounts of Dimethylformamide, Ethanol, or Urea[a]

Pretreatment with DMF, %	Activity/100 mg wet gel, μkat × 10^{-5}	Pretreatment with ethanol, %	Activity/100 mg wet gel, μkat × 10^{-5}	Pretreatment with urea, *M*	Activity/100 mg wet gel, μkat × 10^{-5}
5	3.61	5	2.59	1	2.52
10	3.30	10	2.16	2	2.34
20	4.41	20	3.57	3	2.80
30	5.35	30	4.28	4	5.32
40	3.40	40	2.06	5	4.52
50	2.30	50	3.09	6	3.16
60	3.26	60	3.25	7	2.58
70	3.34	70	2.70	8	2.95
80	3.53	80	2.09		
90	3.40	90	2.58		
100	1.93	100	2.95		

[a]The gels were treated with the organic compounds for 1 day at 37°C and kept for additional 2 days at room temperature. The activity was measured after removal of the dimethylformamide, ethanol, or urea, respectively.

For comparison the same incubations in the presence of dimethylformamide, ethanol, and urea were carried out with 1.4 μkat of native enzyme (Figs. 1–3). In any case, the activity decreased with increasing concentrations of the organic compounds, the only exception being in dimethylformamide, where a slight maximum appeared at 40% of the solvent concentration.

Pretreatment with Dimethylformamide, Ethanol, or Urea

In another series 200 mg aliquots of wet gel were incubated in presence of dimethylformamide, ethanol, or urea, respectively, as described in the previous experiment, but this time the batches were kept at room temperature for additional 2 days. The gels were then removed from the different organic solutions by centrifugation, washed with 0.15*M* KCl solution, and assayed for enzymatic activity. The results were given in Table 2 and Figs. 4–6. All batches tested after pretreatment with the organic compounds showed an increase of activity. The value depended significantly on the concentrations of the reagents used. Pretreatment with dimethylformamide resulted in a large maximum at a concentration of 30%, where the activity could be increased to 3.8-fold that of the untreated gel. A smaller maximum could be observed at a concentration of 80% dimethylformamide with an increase of activity to 2.5-fold. With ethanol, two maxima were also

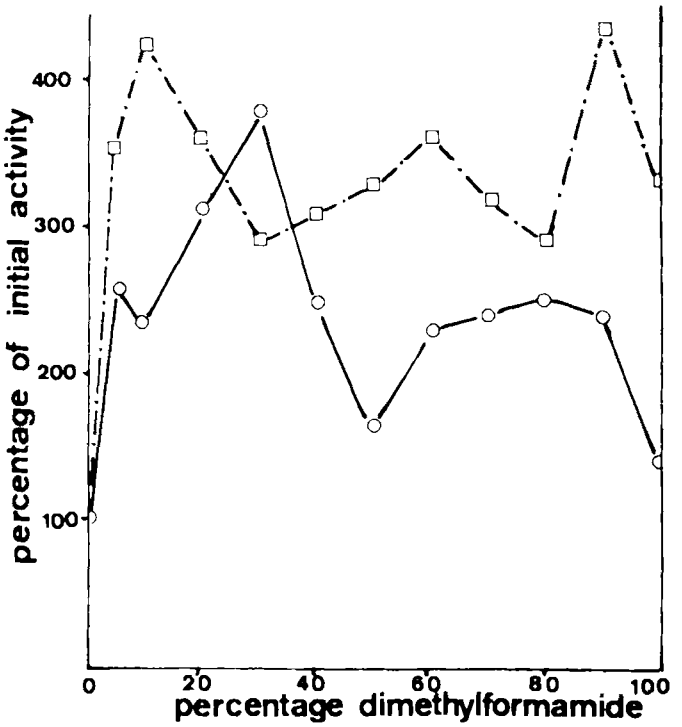

Fig. 4. Activity of immobilized *myo*-inositol-1-phosphate synthase after treatment with dimethylformamide and removal of the solvent: ○—○, assay carried out in absence of soluble NAD^+; □—□, assay carried out in presence of additional soluble NAD^+.

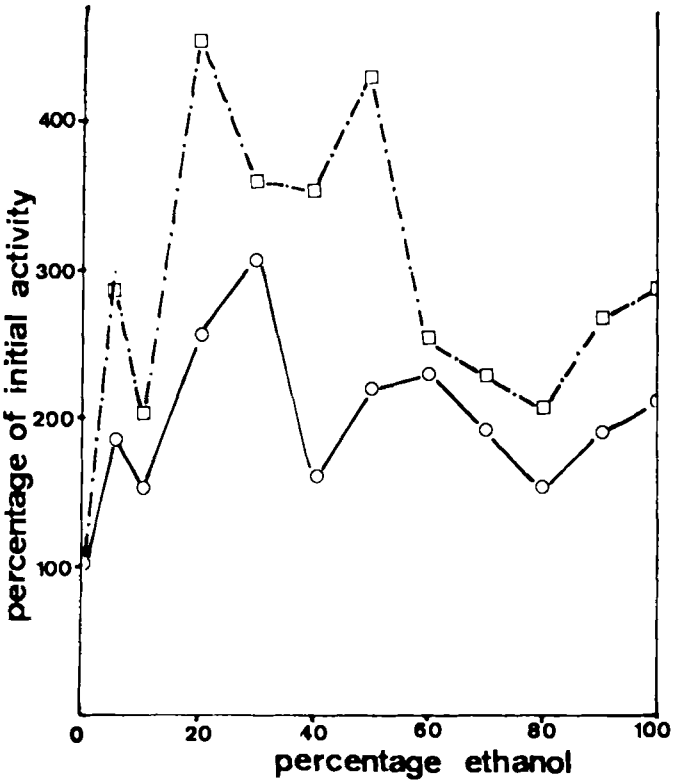

Fig. 5. Activity of immobilized *myo*-inositol-1-phosphate synthase after treatment with ethanol and removal of the solvent: o—o, assay carried out in absence of soluble NAD^+; □—□, assay carried out in presence of additional soluble NAD^+.

observed, the higher at 30% ethanol (a 3-fold increase in activity) and a smaller one at 60% ethanol (a 2.3-fold increase in activity).

With urea, only one significant maximum could be observed when the concentration was 4*M* (a 3.8-fold increase in activity).

Activity of Pretreated Gels after Addition of Soluble NAD^+

The gels mentioned above were assayed for activity a second time, but now in presence of additional soluble NAD^+, the concentration of the incubation mixture being 1 m*M* with respect to this cofactor. Again a significant increase in activity could be observed. In the case of dimethylformamide or ethanol treatment, the positions of the two maxima were different from those observed in absence of soluble NAD^+. With the dimethylformamide-treated gels, the maxima were situated at 10 and 60% (4.2-fold and 3.6-fold activity compared to the untreated gel). With the ethanol-treated gel two maxima at 20 and 50% (activity increased 4.6-fold and 4.3-fold) appeared.

Urea-treated gels showed only one maximum at the same concentration of urea (4*M*) as in absence of soluble NAD^+, the activity reaching a value 5.2-fold of that of the untreated gel.

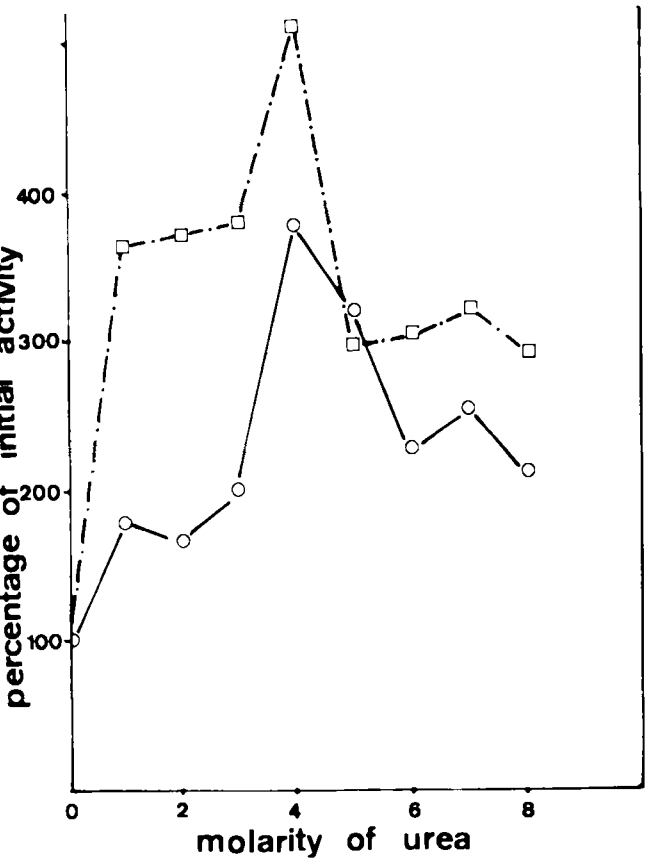

Fig. 6. Activity of immobilized *myo*-inositol-1-phosphate synthase after treatment with urea and removal of the organic compound: o—o, assay carried out in absence of soluble NAD^+; ;b x—□, assay carried out in presence of additional soluble NAD^+.

Discussion

The aim of this paper was to study the influence of some organic compounds on the activity of an enzyme immobilized together with its cofactor on the same matrix, which does not need an additional regenerating system for the cofactor. In most systems published up to now (e.g., *1–5*) enzyme and cofactor are bound to different carriers. Common to all of the systems using NAD^+ as a cofactor was that they need an additional regenerating system, otherwise the reaction stops when all cofactor is used up.

For studies of the influence of organic compounds on the behavior of an immobilized enzyme/cofactor system, it is of interest to immobilize the enzyme together with its cofactor on the same matrix. In addition to that, it is advantageous to achieve independence of an external regenerating system because it is difficult to distinguish between the contributions of the different compartments of such systems on the influence of the organic compounds under investigation. In the case of immobilized cofactor/*myo*-inositol-1-phosphate synthase, we have an ideal system

TABLE 3

Activity of Immobilized NAD^+/*myo*-Inositol-1-phosphate Synthase after Treatment with Increasing Amounts of Dimethylformamide, Ethanol, or Urea[a] when Assayed in Presence of Soluble NAD^+

Pretreatment with DMF, %	Activity/100 mg wet gel, μkat × 10^{-5}	Pretreatment with ethanol, %	Activity/100 mg wet gel, μkat × 10^{-5}	Pretreatment with urea, *M*	Activity/100 mg wet gel, μkat × 10^{-5}
5	4.98	5	3.98	1	5.11
10	5.91	10	2.83	2	5.17
20	5.03	20	6.40	3	5.35
30	4.00	30	5.01	4	7.22
40	4.30	40	4.94	5	4.14
50	4.55	50	6.09	6	4.27
60	5.07	60	3.51	7	4.50
70	4.50	70	3.23	8	4.10
80	4.03	80	2.88		
90	6.10	90	3.70		
100	4.70	100	4.00		

[a]The gels were treated with the organic compounds for 1 day at 37°C and kept for additional 2 days at room temperature. The activity was measured after removal of the dimethylformamide, ethanol, or urea, respectively.

for such studies. It could be shown that immobilization of the enzyme leads to a significant stabilization against denaturation by organic solvents and urea, respectively. It can be seen from Fig. 1–3 that increasing amounts of the various organic compounds present in the incubation mixtures during the enzymatic reaction lead to different behavior of the immobilized enzyme.

With dimethylformamide, the activity increases drastically, reaching a maximum at a concentration of 40% and a second one at 90% dimethylformamide. In the same range the activity of the native enzyme is always lower than in absence of organic solvent, in 90% dimethylformamide it is completely inactive. It is obvious that by immobilization the enzyme is significantly protected against loss of activity. This owes partly to the fact that the tertiary structure of the immobilized enzyme is stabilized and made less flexible by the covalent interaction with the relatively rigid matrix. For interpretation of the peaks, one has to take into consideration that this system consists of crosslinked protein gel whose properties depend on the side groups of the various amino acids forming the protein. Since there exists a difference in the hydrophobic nature of the various amino acid residues, such hydrophobic groups will tend to be buried inside the protein in aqueous solvents. The opposite might occur in presence of organic solvents.

It is already well known (*6*) that with native *myo*-inositol-1-phosphate synthase there exists a very weakly dissociating ternary complex of enzyme, substrate, and cofactor that protects the active site against attack by the solvent. Therefore rearrangement of the protein matrix and the immobilized enzyme as a result of the organic solvents present might occur, leaving the active site unaffected. These facts may account for the peak at 40% dimethylformamide, which could not only be observed with the immobilized enzyme but also with the native form, which seems to develop a second quasi-native state in the presence of 40% dimethylformamide. Such native-like states are already known under certain conditions with some soluble enzymes (*7*, *8*). The reason for the extremly high maximum with the immobilized synthase might be that, in aqueous solutions, the active site is probably not on the surface of the protein, but is exposed to the surface by the action of the organic solvent. For an explanation of the second peak at 90% dimethylformamide (where the native enzyme is completely inactive), there may be several contributory reasons: Even at that concentration of organic solvent, the possible configurations of the immobilized enzyme chains are restricted by the existence of the crosslinks to the matrix, the active site being still protected for the reasons described above. Further, because of its polarity this type of gel contains large amounts of water. Even at that concentration of organic solvent, the water remaining in the gel should suffice for the catalytic reaction. On the other hand, the partition of the very polar substrate glucose 6-phosphate between the gel and the organic solvent-enriched bulk solution should be shifting to the side of the gel, leading to an enrichment of the substrate near the active site. Since the affinity of the substrate for the active site is very high [which cannot be said at all for the product (*9*)] this should cause an increase of activity too.

Comparing the influence of dimethylformamide on the immobilized and the native enzyme with that of ethanol (Figs. 1, 2), one can see striking differences: Besides a slight minimum at 10% ethanol, which might indicate a transition between

two possible conformations of the immobilized enzyme, the activity of the gel remains nearly constant between 0 and 60% ethanol, the activity declining rapidly at higher alcohol concentrations, but never reaching virtually zero.

For comparison, the activity of the native enzyme remains nearly unaffected during the incubation time between 0 and 20% ethanol, gradually declining afterwards, and reaching zero at a content of 80% alcohol in the incubation mixture. The reason for this drastic difference between the action of the two solvents is that ethanol, being far less polar than dimethylformamide, should have a higher affinity to the hydrophobic groups of the crosslinked protein gel than dimethylformamide, thus altering the conformation drastically because the polar groups will now tend to be buried within the macromolecules. Normally the polar groups are situated outside, which seems also to be true in presence of certain concentrations of dimethylformamide.

In presence of urea (Fig. 3) the increase of activity up to a maximum in 3*M* urea (whereas the native enzyme rapidly looses its activity under the same conditions) might result from the fact that the noncovalent bonds in the macromolecules are weakened by the action of urea, giving the immobilized enzyme a chance to rearrange to a more active conformation. With increasing amounts of urea, the protein tends to form random coils without residual noncovalent structure (*10*), the crosslinks being the only bonds preventing the gel from becoming completely randomly coiled. This might explain the small peak at 6*M* urea too. The results show that immobilization can provide a protection even against a denaturing agent like urea, which breaks all noncovalent structures in a protein.

When batches of the gel were treated for several days with increasing amounts of dimethylformamide, ethanol, or urea and assayed for activity after removal of the organic compounds, it turned out that in no case the influence of the organic compound could be reversed to the state of the untreated gel (Figs. 4–6). In all cases an increase of activity could be observed, the value of which was significantly dependent on the concentration of the organic compound used.

Again, peaks of activity could be found, but they differed significantly with respect to position and intensity from those observed when the activity was assayed in presence of the organic compounds. This results from the fact that during the removal of the solvents or urea, respectively, the polar groups of the protein gel are freshly exposed to the surface of the solid phase because the environment becomes more polar. Again, recombinations to different conformations occur; naturally, these must be different from those found in the presence of the organic substances. Obviously, numerous stable conformations seem to exist that are strongly dependent on the pretreatment.

With dimethylformamide-pretreated gel (Fig. 4), no increase of activity after removal of the solvent could be observed. The bigger maximum shifted slightly to the left in comparison to that determined in presence of dimethylformamide. Instead of the sharp peak in presence of 90% dimethylformamide, a broad maximum of decreased activity was observed after removal of the solvent with gel batches pretreated with dimethylformamide concentrations between 60 and 90%.

The behavior with ethanol-treated gels after removal of the various concentra-

tions of the alcohol was quite different. With that series, the activity was strongly increased over the whole range (Fig. 5), forming distinct maxima. It is obvious that ethanol, with its greater affinity to nonpolar groups, changes the behavior of a crosslinked protein gel more drastically than does the more polar dimethylformamide (where the differences in behavior in the presence of or after pretreatment with the solvent do not come out as significantly as with ethanol). Pretreatment with urea (Fig. 6) resulted in an increase of activity over the whole range with a significant maximum at a concentration of 4*M* urea. In that case the enzyme chains seem to be most randomly coiled, whereas the native enzyme is completely denatured. When urea is removed, this batch of gel tends to rearrange to an optimal conformation, leading to the high maximum. At higher urea concentrations for pretreatment, a decrease in activity was observed that might occur because the whole matrix is affected by urea, which might also be visually recognizable: The gels become swollen and evidently looser in their networks even after the removal of urea.

The enzyme assay of the enzyme/cofactor gels pretreated with increasing amounts of dimethylformamide, ethanol, or urea, respectively, were repeated, this time with addition of soluble NAD^+ to the incubation mixtures. Under these conditions, an increase of activity could again be observed. The positions of the maxima of activity were not always correlated to those observed in the absence of soluble NAD^+ (Figs. 4—6). The further increase of activity shows that after the treatment with the organic compounds active sites without immobilized NAD^+ nearby (which were either buried inside the gel or because of a disturbed conformation became less accessible to the substrate) could be transformed to a more active conformation. Therefore, in that case, several parameters are responsible for the high activity: (A) Active sites using immobilized NAD^+ for the reaction and (B) sites that were not active before the action of the organic solvents. The number of these induced active sites again depends on the concentration of the organic solvent used, thus resulting in shifting, and different shapes for the peaks. (Table 3.)

No shifting of the position of the peak could be observed with the urea-treated gels. Here the weakening of nearly all noncovalent bonds by the action of urea does not seem to allow numerous different conformations of the polyprotein after removal of the bond weakening reagent.

In these studies it was demonstrated that optimal coupling conditions do not necessarily lead to the highest obtainable activity of an immobilized enzyme. It is sometimes useful to try to increase the activity of the enzyme again after immobilization. This paper shows that either the presence of polar solvents such as dimethylformamide or a pretreatment with water-miscible solvents of lower dielectric constants such as ethanol, which has a high affinity for hydrophobic groups of the protein might bring good results.

Another good means might be the treatment with agents such as urea or guanidine, which weakens noncovalent bonds. These studies show that there exists an optimal concentration of such reagents, and that this concentration must not be exceeded. These effects will be more significant with flexible and polar gels than with more inert and rigid matrices.

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