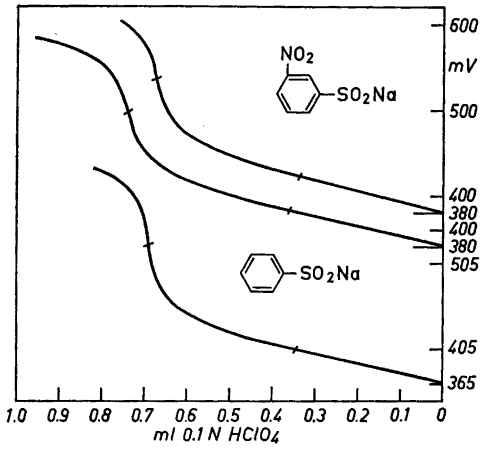

Fig. 2. The titration of sodium benzenesulfinate and sodium *m*-nitro-benzenesulfinate with 0.1 N $HClO_4$ in glacial acetic acid.

duplicate. The reproducibility of the half neutralization potential is indicated by vertical bars in Fig. 1. Fig. 2 shows some typical titration curves.

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Crystallization of Horse Liver Alcohol Dehydrogenase Complexes from Alcohol Solutions

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Large crystals of horse liver alcohol dehydrogenase (LADH) and its complexes with coenzyme and inhibitor molecules were first prepared by Yonetani and Theorell [1] by slow evaporation of concentrated solutions of the protein in weak phosphate buffer at pH 7.0. During a preliminary X-ray investigation [2] of these crystals it was found that they were very fragile and sensitive to small temperature changes. Since they were very unsuitable for accurate X-ray work we have explored other conditions of crystallizations to obtain crystals suitable for a crystal structure determination of LADH. The final conditions reported here were found after many unsuccessful attempts with high salt concentrations, different buffer systems and pH, and various solvents.

Materials and methods. Stock suspensions of microcrystals of homogeneous [3] LADH-e were kept at −20°C in 30 % ethanol-water solution. A suitable amount of this suspension was centrifuged at −20°C and the crystals were dissolved in 0.05 M Tris−HCl buffer at pH 9.4. This and the following treatment were made at +4°C. The solution was then first dialysed for 24 h against 0.05 M Tris−HCl buffer at pH 9.4 and then against 0.05 M Tris−HCl buffer at pH 8.4 containing 5 % of ethanol for another 24 h. Denatured protein was removed by centrifugation and the remaining solution was diluted with buffer to a final protein concentration of 0.5 or 1 % and transferred to dialysis bags each containing 1 ml of protein solution. Each bag was then placed in a bottle containing 20 ml buffer at pH 8.4 and alcohol to a concentration just below the precipitation point. Binary and ternary complexes were prepared by addition of excess coenzyme and inhibitor to the

Table 1.

Complex	Protein conc. in % w/v	Alcohol conc. at precipitation point v/v	Buffer solution	Type of crystals
E	0.05	8	0.5 M TRIS, pH 8.4	A
$E(Phe)_2$	0.05	8	0.5 M TRIS, pH 8.4 + + 0.001 M Phe	A
$E(ADPR)_2$	0.5	9	0.05 M TRIS, pH 8.4 + + 0.001 M ADPR	A
$E(ADPR{\cdot}Phe)_2$	0.5	9	0.05 M TRIS, pH 8.4 + + 0.001 M Phe + + 0.001 M ADPR	A
ER_2	1.0	10.5	0.05 M TRIS, pH 8.4 + + 0.001 M NADH	C
$E(OP)_2$	1.0	11	0.05 M TRIS pH 8.4 + + 0.001 M NAD^+ + + 0.006 M P	C
$E(OPJ)_2$	1.0	11	0.05 M TRIS, pH 8.4 + + 0.001 M NAD^+ + + 0.006 M PJ	C
$E(RI)_2$	1.0	11	0.05 M TRIS, pH 8.4 + + 0.001 M NADH + + 0.006 M I	C
$E(RI)_2$	1.0	13	0.05 M TRIS, pH 8.4 + + 0.01 M K_2HPO_4 + + 0.001 M NADH + 0.006 M I	B
$E(RI)_2$	1.0	11	0.05 M TRIS, pH 8.4 + + 0.0018 M K_2PtCl_4 + + 0.001 M NADH + + 0.006 M I	B

Crystal modifications:
A: orthorhombic symmetry; Space group $C222_1$ $a = 56$ Å, $b = 75$ Å, $c = 181$ Å
B: monoclinic symmetry; Space group $P2_1$ $a = 51$ Å, $b = 44$ Å, $c = 182$ Å, $\gamma = 108°$
C: triclinic symmetry; Space group $P1$ $a = 51$ Å, $b = 44$ Å, $c = 92$ Å, $\alpha = 99.8°$, $\beta = 94.4°$, $\gamma = 105.9°$
Symbols: E = LADH = horse liver alcohol dehydrogenase; O = NAD^+ = nicotinamide-adenine-dinucleotide; R = NADH; ADPR = Adenosine diphosphate ribose; Phe = *o*-Phenantroline; I = Isobutyramide; P = Pyrazole; PJ = 3-Iodopyrazole.

protein solution prior to the dialysis procedure. The complexes were then dialyzed during crystallization against buffer containing excess coenzyme and inhibitor.

By successive small additions of alcohol the alcohol concentration was increased until precipitation started. Ethanol was used in the crystallization of all complexes except those containing NAD^+ where methanol was used.

The crystals were allowed to grow for several days at this alcohol concentration. Further growth without the formation of new crystals

could then be achieved by successively increasing the alcohol concentration another 5–10 %.

The crystals obtained in this way are large but fairly fragile and dissolve in their mother liquor at room temperature.

Results. Table 1 lists the various complexes investigated, their conditions of crystallization and the types of crystals obtained. All X-ray exposures have been made at +4°C using copper radiation to take precession photographs. The diffraction pattern extends to a resolution of at least 2.5 Å but there is a sharp decrease in the intensities of reflexions beyond 2.8 Å. Fig. 1 shows a copy of an 18.5° precession photograph of *hhl* reflexions of type C crystals.

It is interesting to note that the so called "mosaic" complex $E(ADPR{\cdot}Phe)_2$ first described by Yonetani [4,5] crystallizes isomorphous to free LADH whereas all complexes involving the coenzyme crystallize in different modifications. Since recent kinetic studies of LADH [6] (stopped flow) have shown that an intramolecular rearrangement of the binary complex is necessary for the action of the enzyme, these results indicate that the whole coenzyme is required for this conformational change.

It was previously reported [2] that crystals of the binary complex ER_2 obtained by evaporation of a phosphate buffer solution were isomorphous to crystals of free LADH. These crystals were, however, in all probability composed of free LADH since we know that our enzyme preparations at that time contained impurities which caused a decomposition of the coenzyme. We have repeated this crystallization using enzyme preparations without these impurities and examined the crystals. It has been found that these ER_2 crystals were of type B, thus different from free LADH and isomorphous to $E(RI)_2$ crystals prepared in the presence of K_2HPO_4.

Type B crystals of $E(RI)_2$ are also formed in the presence of K_2PtCl_4. These crystals are isomorphous to those obtained with K_2HPO_4 and we are now analysing the intensity differences obtained in the hope that this is a suitable heavy-atom derivative for type B-crystals.

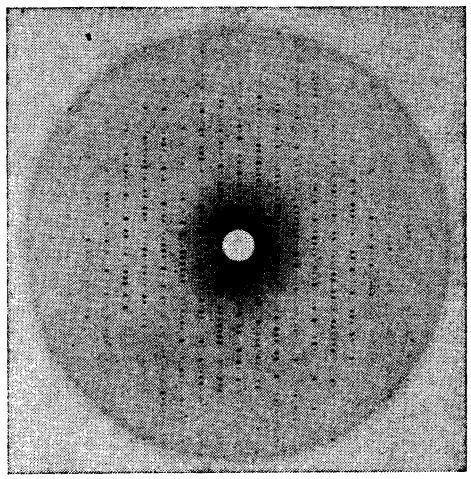

Fig. 1. 18.5° precession photograph of *hhl* reflexions from type C crystals of LADH recorded at +4°C.

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