MEETING REPORT

PYRIDINE NUCLEOTIDE-DEPENDENT DEHYDROGENASES A REPORT OF AN ADVANCED STUDY INSTITUTE HELD AT THE UNIVERSITY OF KONSTANZ, GERMANY, SEPTEMBER 15–20, 1969*

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Received 5 May 1970

Introduction

In recent years there has been considerable progress in the elucidation of the enzymic properties and the structure of pyridine nucleotide-dependent dehydrogenases. Though many of those working in this field have realized for a long time, the need for a detailed discussion of the mechanism of action of these enzymes, this field, unfortunately and in contrast to many others, has never been dealt with in a symposium. Unresolved problems and contradictory results require a discussion in a symposium which allows personal contact. Therefore, a symposium on pyridine nucleotide-dependent dehydrogenases was held on the campus of the recently established University of Konstanz.

The aim of the symposium was to provide a forum for a discussion among experts interested in the various aspects of dehydrogenases and pyridine coenzymes, so as to evaluate the state of our present knowledge and to stimulate further progress in this field. 90 Participants including the invited speakers came to the symposium. All participants had received the printed lectures beforehand and, therefore, the emphasis during the symposium was on discussions. The programme for the symposium was elaborated by an organizing committee consisting of Keith Dalziel, Sir Hans Krebs, Horst Sund, Cornelius Veeger, and Kurt Wallenfels. The symposium was sponsored by the *International Union of Biochemistry*. A great deal of financial support was given by the *NATO Science Committee* under its *Advanced Study Institute Programme* and by the *Gesellschaft der Freunde und Förderer der Universität Konstanz*. The programme was divided into eight sections:

1. General

In the first lecture K.Dalziel (Oxford) discussed the general kinetic aspects of reactions catalyzed by simple oxidoreductases and those in which three substrates are involved. The mechanism of the hydrogen transfer from the chemical point of view was treated by K.A.Schellenberg (Baltimore) and K.Wallenfels (Freiburg). It is now generally assumed that the hydrogen transfer catalyzed by pyridine nucleotide-dependent dehydrogenases proceeds via a direct transfer of a hydride ion. Although hydride transfer implies the simultaneous transfer of a proton and two electrons, the term "simultaneous" only means that no detectable time difference exists in the transfer of the two electrons. The quantum-mechanical probability of two electrons moving at exactly the same time is nearly zero. However, since the nucleus moves so much more slowly than do the electrons, one of the electrons can accompany the proton, with the other preceding it or following it, and still be "simultaneous" as far as practical determination of the mechanism is concerned.

During the enzymic reaction the methylene groups

* The complete proceedings have been published in book form, *Pyridine Nucleotide-Dependent Dehydrogenases*, ed. H.Sund, Springer-Verlag, Berlin-Heidelberg-New York, 1970.

of tryptophan residues are labelled when tritium-labelled substrates are used. This was considered compatible with the idea that tryptophan participates as an intermediate in the enzymic reaction. The mechanistic significance of this transfer is not known with certainty; the tryptophan may be an intermediate or it may act in a side reaction unrelated to the actual enzyme mechanism. The X-ray analysis of dogfish lactate dehydrogenase shows that a tryptophan residue is not located at or near the coenzyme-binding site and, therefore, it seems improbable that tryptophan is an intermediate.

NMR Spectroscopy has been used for the elucidation of the conformation of pyridine coenzymes (N.O. Kaplan, San Diego). In the folded form of NAD and NADP, the purine interacts with the side of the pyridine ring containing the amide group at C_3. There is much more deshielding of the pyridine ring in NADPH than in NADH. The bulky monoester phosphate group in NADPH tends to separate the purine from the reduced pyridine ring. The conformation of the purine ribose is not influenced by reduction, but there is a marked alteration in the geometry of the pyridine ribose. This causes an alteration of the diphosphate backbone and, therefore, a different geometry between the reduced pyridine and adenine moieties as compared to the interaction of the bases in the oxidized coenzyme. The pyridine coenzymes probably exist in three structures: an open form and two folded forms; the latter are distinguished by the relative positions of the pyridine and purine rings and have been referred to as the right and left helices. The two hydrogens of the reduced pyridine at C_4 exist in different environments in the two folded forms. The difference in stereospecificity with respect to the coenzyme of the A and B dehydrogenase may be related to their capacities to recognize one of the two helices. The two helical forms are in equilibrium with each other and their interconversion proceeds probably through an open form.

2. Structure and function of dehydrogenases

A comparison of GAPDH† from pig and lobster muscle (332 and 333 amino acids, respectively) shows that 241 (72%) of the residues occur in identical sequence (J.I.Harris, Cambridge). Moreover, a comparison of the pig enzyme with the available sequence information on the rabbit and beef muscle enzymes demonstrates that the three mammalian enzymes are almost identical in sequence, while a provisional comparison of the yeast (331 amino acids) and mammalian enzymes shows a sequence homology of approximately 67%.

The –SH groups of dehydrogenases so far investigated react stereoselectively with α-iodopropionic acid or with its amide (B.Eisele and K.Wallenfels, Freiburg). The A specific enzymes are inactivated preferentially by the D antipodes, whereas the B specific enzymes react faster with the L antipodes. This result reflects common structural features in the active sites of the A (or B) specific enzymes. It is, however, still questionable whether the regularities found can be generalized.

The role of zinc ions in the mechanism of action of dehydrogenases was discussed intensively (T.Keleti, Budapest). It was agreed that alcohol dehydrogenases are zinc enzymes, while all the other dehydrogenases investigated, with the exception of glyceraldehyde-3-phosphate dehydrogenases, are not. Regarding the latter the discussion was controversial. Zinc can be partially removed from the rabbit enzyme with parallel loss of activity whereas the lobster enzyme does not contain zinc. With respect to zinc, GAPDH from different sources might be different as are aldolases. Aldolase from yeast is a zinc enzyme whereas the muscle enzyme does not contain zinc though the amino acid analysis of these two enzymes is very similar.

3. Alcohol and lactate dehydrogenase

ADH from horse liver is composed of two polypeptide chains which can be different: "E" with activity after dimerization only towards ethanol, but none towards steroids, and "S" with weaker activity towards ethanol, but with full activity towards steroids (H.Theorell, Stockholm). The sequence of the

† *Abbreviations:*
ADH : alcohol dehydrogenase.
GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
GluDH : glutamate dehydrogenase.
LDH : lactate dehydrogenase.

370 ± 10 amino acids has now been almost completely established and shows differences in five positions between E and S. Both E and S can exist in subfractions (E′ and S′), so that a total of ten isoenzymes could be expected. So far nine isoenzymes have been found. E can be transformed to E′ and the cause of this transformation may be the loss of amide groups.

Investigations of LDH (G.Pfleiderer, Bochum, and G.Schwert, Lexington) have shown that guanidation causes loss of activity without alteration of enzyme-NADH binding and that the same isoenzymes in different species are probably more closely related than different isoenzymes from single species.

Two participants came to Konstanz with a lot of baggage: C.-I.Brändén (Uppsala) and M.G.Rossmann (Lafayette). It was a high-point of the Konstanz meeting when the models of liver ADH at 6 Å resolution and of dogfish LDH at 5 Å resolution were presented side by side. The availability of both models at the same time gave an opportunity to search for possible similarities in their structure. The first comparisons were based on the superposition of the ADH dimer axis on each of the LDH two-fold axes in turn. No resemblance was apparent between these dimers. However, inspection of the individual monomer subunits (polypeptide chains) quickly revealed some basic resemblances (fig. 1). Both subunits had a significant cleft and a characteristic wing. When these were superimposed, the remainder of the subunits had essentially the same overall dimensions. These similarities were further enhanced by reallocating some of the electron density across the ADH molecular two-fold axis. Individual chains could be compared in many parts of both structures. Furthermore, the known coenzyme binding site of LDH was in the vicinity of one of the two sites associated with an ADH mercury-labelled NADH-competitive inhibitor. Recognition of the dissimilar subunit contact areas leads to the speculation that the dehydrogenase subunit is more highly conserved than the active dimer (ADH) or tetramer (LDH). Presumably association into higher oligomers is a more recent event in the evolution of each molecule.

The general shape of the ADH molecule is ellipsoidal 40 × 50 × 100 Å along the a, b, and c directions, respectively. The position of the dialyzable zinc is probably at the bottom of the large cleft in each subunit. The position of the other zinc is not known. The coenzyme binding sites are located on the surface and about 75 Å apart in the dimer. The identified zinc position is more than 25 Å from the coenzyme binding site. This zinc, therefore, is probably not involved in the active centre; its function is not known.

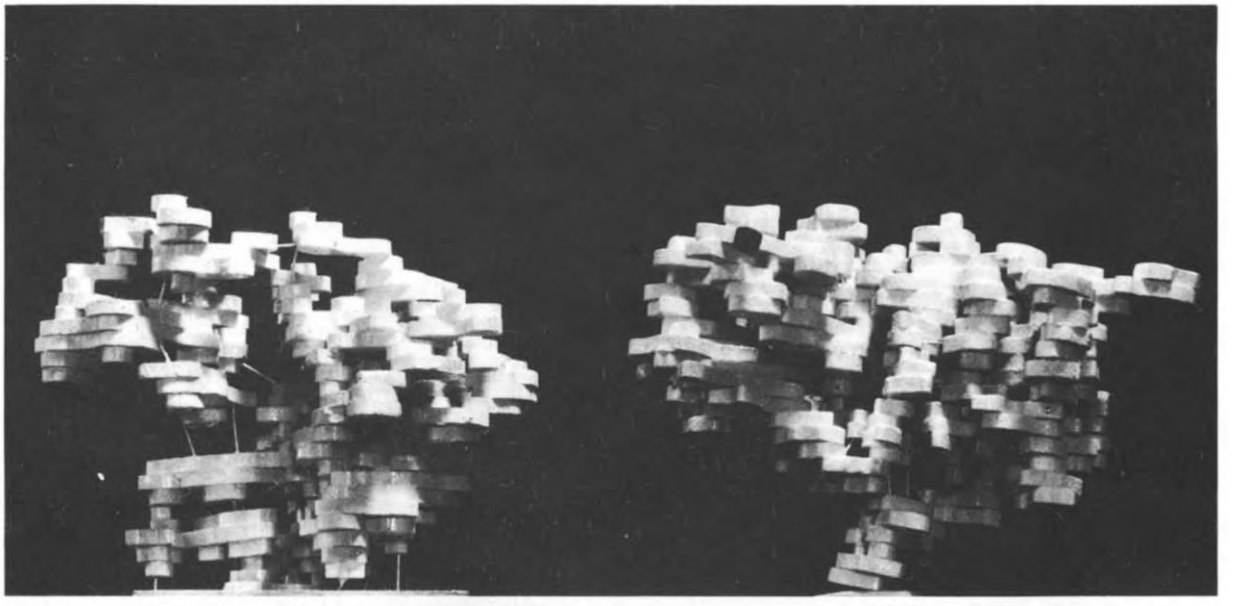

Fig. 1. Models of the monomer subunits (polypeptide chains) of horse liver ADH based on the 6 Å resolution (Brändén and his coworkers, left) and dogfish M_4 LDH based on the 5 Å resolution (Rossmann and his coworkers, right).

The coenzyme binding sites in LDH are also relatively far from each other, 21 Å on the same side of the molecule, and the closest approach on opposite sides of the molecule is 54 Å. Upon coenzyme binding, a change of the crystal symmetry and of the conformation is observed. Not only the tertiary structure of each individual polypeptide chain, but also the relative position of the chains are changed. AMP is the smallest part of the coenzyme molecule which causes conformational change upon binding, for instance adenosine also binds but does not change the conformation. The rate controlling step in the reaction catalyzed by LDH is probably the conformational step. The coenzyme molecule is bound to the enzyme in a wide open structure. The binding studies suggest that the nicotinamide end of NAD cannot bind unless the adenine end has first produced the conformational change. The "essential" –SH group is almost at the centre of the subunit and not located in the active centre. Therefore, it appears that the essential –SH group is only essential to maintain that conformation which is necessary for enzymic activity.

4. Glyceraldehyde-3-phosphate dehydrogenases

Six papers were devoted to the discussion of the mechanism of GAPDH. P.Elödi (Budapest) had investigated the selective reactivity of cysteinyl, tryptophanyl, and tyrosyl groups. The reactivity of –SH groups of the rabbit muscle enzyme had been intensively studied by S.A.Bernhard (Eugene) with regard to the elucidation of the mechanism of enzyme-substrate covalent bond formation during the enzyme-catalyzed reaction. The results of inhibition with iodoacetate or iodoacetamine are consistent with a model in which there are four *identical* thiol active sites. However, the catalytic reactivity with the acylating reagent β-(2-furyl)acryloyl phosphate is not consistent with this model. This reaction is complete when two acyl groups have reacted with the tetrameric enzyme molecule. From the study of preparations with different degrees of alkylation or acylation, it was suggested that both coenzyme binding *and* acylation significantly affect the symmetry restrictions on the subunits in the enzyme tetramer.

In contrast to the work of Kirschner and Eigen on yeast GAPDH, D.E.Koshland (Berkeley) presented data on this enzyme which shows non-identity of the coenzyme binding sites. The binding of NAD has been examined by equilibrium dialysis and steady-state kinetics. The results are explained on the basis of ligand induced changes of subunit conformation caused by the binding of NAD^+ and indicate a combination of positive and negative cooperative effects. The association constants for the four NAD^+ binding sites ($K_1 = 1.8 \times 10^4\ M^{-1}$, $K_2 = 2.1 \times 10^5\ M^{-1}$, $K_3 = 4.9 \times 10^4\ M^{-1}$, and $K_4 = 8.8 \times 10^2\ M^{-1}$) indicate positive cooperativity between the first and second binding sites, but negative cooperativity between the second and third and third and fourth binding sites. The binding of the first NAD^+ molecule appears, therefore, to make it easier for the second molecule to be bound. However, after the second molecule is bound, the binding of the third NAD^+ molecule is made more difficult and a similar negative effect is extended to the binding of the fourth coenzyme molecule. A vivid discussion between Dalziel, Eigen, and Koshland after the lecture was not able to clarify the discrepancies of the results which support either the Monod model (Eigen) or the model proposed by Koshland.

Hydrodynamic, spectroscopic, and X-ray small-angle measurements are in accord with the predictions of the concerted or allosteric mechanism. NAD^+ binding is accompanied by an increase in the diffusion and sedimentation coefficients. Upon NAD^+ binding, optical rotatory dispersion and circular dichroism show parallel changes which are shown to be conformational in origin, rather than a consequence of NAD^+ attachment *per se*. The experimentally found relationship between conformational changes and degree of ligand saturation is in agreement with the relationship calculated for a two-state model ($R \rightleftharpoons T$) (R. Jaenicke, Frankfurt). The structural changes observed by X-ray small-angle measurements upon NAD^+ binding show the same relationship (I.Schuster, Göttingen). The transition from the less affine and enzymic inactive T-state to the active R-state is accompanied by an increase of anisotropy and a volume contraction of 7%. In addition, the NAD^+ binding at different pH values and temperatures can be explained in full accord with the allosteric model involving the isomerization step $R \rightleftharpoons T$. Although no change in the quaternary structure occurs on NAD^+ binding, citrate at

high concentration (up to 1 M) causes dissociation without loss of enzymic activity (I.Schuster, Göttingen). The dissociation product has a molecular weight of about 40 000 indicating that subunits are formed which contain two polypeptide chains compared to four in the "native" molecule. Spectrophotometric titrations show that the affinity for NAD^+ was not only unaffected but actually increased in the presence of citrate.

The binding of NAD^+ to GAPDH from rabbit muscle shows negative cooperativity for all coenzyme binding sites ($K_1 < K_2 < K_3 < K_4$) (J.J.M.de Vijlder, Amsterdam). From kinetic experiments, it was concluded that in the oxidation of glyceraldehyde by NAD^+, the fourth NAD^+ binding site is catalytically more active than the other three. Apart from its greater instability and some differences in kinetics, the properties of the lobster-muscle and the rabbit-muscle enzyme are similar.

5. Glutamate dehydrogenase

A whole day was devoted to GluDH, one of the most fascinating enzymes. This enzyme differs from the other dehydrogenases in many respects: (a) the dehydrogenation occurs at a C–N function of the substrate molecule instead of a C–O function, (b) the molecular weight is high, (c) the enzyme protein exists in an association-dissociation equilibrium with subunits which are all enzymically active, the molecular weight of the smallest enzymically active subunit ("oligomer") was found to be about 300 000, and (d) the enzymic properties and the structure are influenced by a variety of effectors.

A detailed reinvestigation of the kinetics by P.C. Engel and K.Dalziel (Oxford) was made with wide concentration ranges of the coenzymes NAD^+ and $NADP^+$ and the substrates glutamate and norvaline. Deviations from Michaelis-Menten behaviour towards higher activity were observed with increasing concentrations of either coenzyme with glutamate, but not with norvaline as substrate. In phosphate buffer, pH 7.0, Lineweaver-Burk plots with either coenzyme as variable, and a constant, large glutamate concentration showed three or four linear regions of different slope with relatively sharp discontinuities. The results are interpreted in terms of negative homotropic interactions between the polypeptide chains in the oligomer. It is suggested that sharp discontinuities in Lineweaver-Burk plots or reciprocal binding plots may be characteristic of this new type of interaction which can be explained in terms of an Adair-Koshland model, but not by the model of Monod-Wyman-Changeux. In contrast to the results obtained earlier by Frieden, a compulsory order steady-state mechanism can be ruled out.

Optical probes were used for the investigation of the allosteric properties of GluDH by G.K.Radda and his group (Oxford). From fluorimetric measurements in the presence of 1-anilino-naphthalene-8-sulphonate, 2-(*N*-methylanilino)-naphthalene-6-sulphonate, tetranitromethane and 4-iodoacetamidosalicylic acid, it was possible to demonstrate and to characterize conformational changes concomitant with GTP inhibition. Nitration of tyrosyl groups with tetranitromethane abolishes the sensitivity of GluDH to the allosteric inhibitor, GTP, but does not affect the enzymic activity and the hydrodynamic properties. Fluorescence measurements in the presence of 1-anilino-naphthalene-8-sulphonate suggest that the ADP and GTP binding sites are distinct and that ADP and NAD sites are closer than the GTP and NAD sites.

NADH stimulates the inactivation of GluDH (B. Eisenkraft, and C.Veeger, Wageningen). On the basis of the available evidence, the process of inactivation is characterized by a combination of conformational changes. Associated particles dissociate first into the fully active oligomers, which are then gradually converted into a less active or inactive conformation. Obviously associated particles are not inactivated directly.

The structure of GluDH in solution has been studied by the Konstanz group (J.Krause, K.Markau, M. Minssen and H.Sund). X-Ray small angle and other data show that the GluDH molecules have a prolate shape and that dissociation into oligomers involves a transverse cleavage. The radius of gyration of the cross-section and the mass per unit length were independent of protein concentration and, therefore, independent of molecular weight. The results obtained by light scattering experiments clearly show that the association-dissociation equilibrium which is observed with GluDH can be described as an open association-dissociation equilibrium with the consecutive association of monomers without limit and with identical equilibrium constants for all steps. The dependence

of the molecular weight on protein concentration reaches a maximum at about 9 mg/ml. Since the decrease of the molecular weight at higher protein concentrations cannot be ascribed to equilibrium constants which are always positive, the decrease is due to effects of non-ideality. The experimental data are consistent with a second virial coefficient (A_2 = 8×10^{-9} [mole. l.g^{-2}]). A closed association-dissociation equilibrium without any intermediates can be excluded. Both the structure and the catalytic properties of GluDH are altered by a variety of small molecules. ADP was found to stabilize a conformation which shows a higher enzymic activity. The activation is pH dependent. At alkaline pH values ADP stimulates to a greater extent (about fourfold) than at pH 7.6. In addition, GluDH is protected by ADP against denaturation at pH 10.6 where the activity of the enzyme in the absence of ADP is irreversible and completely lost. These effects cannot be attributed to ionic effects which occur through binding of ADP to functional groups of the enzyme protein.

The properties of GluDH in solution have also been studied by H.Eisenberg (Rehovot). Based on light-scattering measurements, electron micrographs and Sund's X-ray small-angle measurements, a model of the oligomer was proposed. In this model the oligomer is formed from two layers, each composed of three polypeptide chains (molecular weight 52 000) arranged in a triangular fashion. The two layers, one stacked on top of the other, form a prolate oligomer which associates with others forming rodlike particles of indefinite length in the direction of the major axes of the oligomer. In electron micrographs, long particles of length more than 1000 Å can be observed directly, beside the monomers. The concentration dependent association is inhibited in the presence of GTP and NADH. Toluene, in constrast to GTP and NADH, enhances the association drastically. Dissociation into active half-oligomers (one layer) containing three polypeptide chains has not been achieved.

Dinitrophenylation of GluDH causes desensitization to ADP and GTP (G.di Prisco, Naples). The data indicate that that each polypeptide chain has on average one lysyl and one half tyrosyl residue associated with catalytic activity, allosteric activation and allosteric inhibition. Although the subunits may be identical in terms of primary structure, it follows that they may not be equivalent in terms of function (due, for example, to the quaternary structure of the oligomer), since only one tyrosyl residue per pair of polypeptide chains appears to be necessary for the reactivity of each type of site.

6. Different aspects of reactions catalyzed by dehydrogenases

In contrast to the NAD-dependent isocitrate dehydrogenase, the NADP-dependent enzyme does not exhibit "regulatory" properties. Kinetic studies on the NADP enzyme (K.Dalziel, Oxford) do not lead to any firm conclusions about the reaction sequence, except that it involves ternary and quaternary complexes. Some data indicate a random order mechanism.

The oxidative decarboxylation of UDP-D-Glucuronic acid, the last step in the biosynthesis of UDP-D-Xylose, has been investigated by D.S.Feingold (Pittsburgh). After binding of UDP-D-glucuronic acid to the enzyme-NAD^+ complex, NAD^+ is reduced and the 4-keto intermediate decarboxylates in an irreversible step leaving a carbanion at C-5. During enolization of this compound, C-5 would assume a planar configuration. Inversion of configuration would occur with the incorporation of the proton at C-5 to form UDP-4-keto-xylose. The incorporation of the proton must occur while the substrate is still enzyme-bound because of the complete stereospecificity of the reaction. The reduction at C-4, involving NADH and the hydrogen originally removed from C-4, is probably the terminal step in the reaction and appears to be irreversible. The stereospecific incorporation of the proton at C-5 may be enzyme-mediated or simply due to steric hindrance by the enzyme molecule.

Transhydrogenases from *Azotobacter vinelandii* and from *Pseudomonas aeruginosa* were described by H.W.J.van den Broek and C.Veeger (Wageningen) and by N.O.Kaplan (La Jolla). The enzymes were purified, characterized and compared. The two enzymes have somewhat related structures, i.e. high molecular weight (sedimentation coefficients 70–120 S). However, the activation by 2'-AMP is different.

7. Pyridine nucleotide-dependent flavin enzymes

Three lectures in this section dealt with complex pyridine nucleotide-dependent dehydrogenases*. J. Visser and C.Veeger (Wageningen) presented data on the role of NAD^+ in the catalytic mechanism of lipoamide dehydrogenase. T.P.Singer (San Francisco) discussed the NADH dehydrogenase of the respiratory chain and V.Massey (Ann Arbor) presented evidence for a new intermediate seen in four different flavoproteins (old yellow enzyme, ferredoxin-TPN-reductase, *p*-hydroxybenzoate hydroxylase, and thioredoxin reductase) on reaction with the reducing substrate, NADPH. The very similar spectral intermediates in the reaction of four different flavoproteins with NADPH is great interest, especially when one considers the very different nature of flavoproteins and the reactions they catalyze.

8. Metabolic aspects

The last section dealt with metabolic aspects. H.A. Krebs (Oxford) discussed the regulation of the redox state of the pyridine nucleotides in rat liver intensively. He found that the ratio [ATP]/[ADP] [P_i], as measured by direct determination of the three components in the liver, and approximately the same value as the ratio calculated from the concentrations of lactate, pyruvate, glyceraldehyde phosphate and 3-phospho-glycerate on the assumption that LDH, GAPDH and 3-phosphoglycerate kinase are at near-equilibrium. This means that the redox state of the NAD couple in the cytoplasm is linked to, and partially controlled by, the phosphorylation state of the adenine nucleotides, The redox state of the cytoplasmic NADH-couple is linked to that of the cytoplasmic NADP-couple by equilibrium reactions in which malic enzyme and LDH or isocitrate dehydrogenase, malate dehydrogenase and glutamate oxaloacetate transaminase and others are involved. It is likely that the redox state of mitochondrial NAD couple is regulated in an analogous manner, though the evidence in support of this view is only suggestive.

The main feature emerging from the analysis is a network of near-equilibria in which the pyridine and adenine nucleotides are the key reactants. This network establishes basal levels of the redox state of the two pyridine nucleotide couples in the two main cell compartments where the energy transforming mechanisms are located and links the redox states to the supply of ATP. It sets the cytoplasmic level of the NAD-couple to be suitable for both glycolysis and gluconeogenesis; it sets the cytoplasmic redox state of the NADP-couple at a more reduced level so as to be effective in reductive synthesis; it sets the mitochondrial redox state of the NAD-couple to be suitable for efficient oxidative phosphorylation.

The state of the NAD system in the liver was also analyzed by Th.Bücher (München). He studied the intercellular compartmentation of pyridine nucleotides ("spatial compartmentation") in experiments with hemoglobin-free perfused rat liver during anoxia and ethanol cycle. However, not only the spatial compartmentation of the pyridine nucleotides determines the actual potential ("Ist-Potential") in the cell but also the binding of the pyridine nucleotides to dehydrogenases. The binding sites with different affinities for the oxidized and reduced pyridine nucleotides in the spatial compartments cause a "compartmentation by binding". These problems indicate that the redox state of the NAD system in intact liver cells provides a classical example for the necessity of distinguishing between the tissue level of a metabolite and its effective concentration in a distinct cellular space. For the analysis of these problems two methods, the *redox metabolite indicator method* and *surface fluorometry*, were used which are more specific than those offered by quenching, grinding, and extraction of the tissue.

* cf. also P.Hemmerich, G.Nagelschneider, and C.Veeger, FEBS Letters 8 (1970) 69.