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Pyridine–Adenine Dinucleotide Transhydrogenase Activity in Cells Cultured from Rat Hepatoma[1]

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Preparations from cells cultured from a minimal-deviation hepatoma in the rat exhibit pyridine nucleotide transhydrogenase (NAD(P)H:NAD(P) oxidoreductase, EC 1.6.1.1) activity. The pH optimum, its release by digitonin, and its apparent lack of dependence on steroids for activity tentatively classify it as a transhydrogenase of the type first described for animal tissue.

Enzyme preparations from digitonin-treated homogenates were very unstable. The time necessary for the loss of one-half the activity was 16–18 h when the enzyme was stored at 5 °C; this was reduced to 4 h when storage was in polycarbonate tubes.

The enzyme apparently transferred hydrogen directly and with equal ease from NADH to both the 3-acetylpyridine and thionicotinamide analogues of NAD. Half-saturation values for NAD and its acetylpyridine analogue were 0.99×10^{-5} *M* and 3.55×10^{-4} *M*, respectively. The enzyme exhibited its maximum activity in phosphate buffer at pH 5.8. It was inhibited by 50–60% over the pH range 7.0–8.5 in Tris buffer. This could be reversed by dithiothreitol; reversal was complete between pH 8.0 and 8.5.

DE LUCA, C., et GIOELI, R. P. Pyridine–adenine dinucleotide transhydrogenase activity in cells cultured from rat hepatoma. Can. J. Biochem. **50**, 447–456 (1972).

Nous avons décelé l'activité d'une pyridine nucléotide transhydrogénase (NAD(P)H:NAD(P) oxydoréductase, EC 1.6.1.1) dans des préparations de cellules cultivées à partir d'un hépatome du type "minimal-deviation" chez le rat. A cause de son pH optimum, de sa libération par la digitonine et du fait que son activité ne semble pas dépendre des stéroïdes, nous suggérons de classifier cet enzyme comme une transhydrogénase du type d'abord décrit pour les tissus animaux.

Les préparations enzymatiques provenant d'homogénats traités à la digitonine sont très instables. L'enzyme perd la moitié de son activité en 16–18 h quand il est gardé à 5 °C; ce temps est réduit à 4 h si l'enzyme est gardé dans des tubes de polycarbonate.

Il semble que l'enzyme transfère directement et avec une égale facilité l'hydrogène du NADH sur les deux analogues 3-acétylpyridine et thionicotinamide NAD. Les valeurs de demi-saturation pour le NAD et son analogue acétylpyridine sont respectivement de 0.99×10^{-5} *M* et 3.55×10^{-4} *M*. L'activité de l'enzyme est maximum dans un tampon phosphate à pH 5.8. Dans le tampon Tris, entre les pH 7.0 et 8.5, l'enzyme est inhibé de 50–60%. Cette inhibition peut être renversée par le dithiothréitol; le renversement est total entre les pH 8.0 et 8.5.

Introduction

Solubilized preparations from beef heart were shown by Kaplan *et al.* (1) to catalyze the transfer of hydrogen between reduced nicotinamide–adenine dinucleotide (NADH) and nicotinamide–adenine dinucleotide phosphate (NADP) according to the reversible reaction NADH + NADP $\rightleftharpoons$ NAD + NADPH. Subsequent studies in the same laboratory (2) showed this transhydrogenase (NADH:NADP oxidoreductase, EC 1.6.1.1) reaction to occur more generally between these nucleotides and certain of their analogues.

The prediction (1) that this activity was widespread was soon confirmed by others (3, 4); highest levels of activity were found in the

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mitochondria of heart, kidney, and liver of a variety of species of animals. The enzyme has also been demonstrated in malignant cells (2, 5); Evans and Kaplan (5) have shown it to be significantly elevated in both myeloid and lymphocytic leukemias, especially in their acute forms.

Intense interest in the nature and physiologic role of this activity led to the discovery of other types of enzymes possessing transhydrogenase (TH) capabilities. Soluble preparations have been isolated from certain endocrine-sensitive tissues. These are apparently of two different classes: (*a*) an estrogen-dependent transhydrogenase, such as that described by Hagerman and Villee (6), and (*b*) a steroid dehydrogenase such as that described by Talalay and Williams-Ashman (7).

In 1961, Klingenberg and Schollmeyer (8) and Estabrook *et al.* (9) reported an energy-controlled reduction of endogenous NADP by NADH in intact mitochondria. This was followed by the work of Estabrook and his collaborators (10) and of Danielson and Ernster (11) on ATP-stimulated TH activity in submitochondrial particles. This was finally confirmed in Kaplan's laboratory (12).

We wish to report here some characteristics of transhydrogenase activity found in preparations from rat hepatoma cells cultured *in vitro*.

Materials and Methods

Cell Cultures and Methodology

The studies reported here were performed with cell line H4-II-E-C3, derived from a minimal-deviation hepatoma (13). Full details of the methods used for growth and maintenance of these cells have been published elsewhere (14). For these studies, however, a trypsin–EDTA mixture (0.20%–0.02%) was used for harvesting cells in place of trypsin alone; monolayers of cells were grown in stationary flasks rather than in roller bottles.

Enzyme Preparation

Growth medium from late log-phase cultures was decanted, and monolayers of cells were rinsed twice each with 5 ml of 0.85% saline and once with trypsin–EDTA mixture. The cells were then freed from the glass substratum by treatment with 2.5 ml of trypsin–EDTA for 10 min at room temperature. Released cells were transferred to a 40 ml centrifuge tube on ice with the aid of several washes of ice-cold saline, and recovered by centrifugation at 750 *g* for 8 min at 5 °C. The supernatant fluid was discarded and the pellet of cells was transferred to a glass Potter–Elvehjem homogenizer tube with the aid of 5–10 ml of cold saline. The pellet was vortexed gently and the cells were sedimented by centrifugation as before. The washed-cell harvest was finally resuspended in 0.005 *M* phosphate buffer (1 ml/approximately 10^7 cells), pH 7.2, and stored in the deep freeze at −15 °C.

At the time of assay, the cell preparation was thawed and homogenized. Neutralized digitonin was added (0.3 ml of 1% digitonin/0.9 ml of cell lysate) and the mixture was allowed to stand on ice for 15 min. The digitonin-treated cell preparation was then centrifuged at 800 *g* for 10 min at 5 °C. The clear, colorless supernatant fluid was decanted and used as the TH preparation. In some cases the digitonin treatment was omitted, as noted under Results.

Transhydrogenase Activity

The measurement of TH activity was essentially that described by Stein *et al.* (2), and was based on the reduction of two specific analogues of NAD in the presence of reduced NAD and the digitonin-treated cell preparation described above. The reaction mixture contained 200 μmol of buffer, water, about 1.00 μmol of analogue, 0.15 μmol of dithiothreitol (DTT), 0.1 ml of TH preparation (containing about 100 μg of protein), and 0.26 μmol of NADH, in a total volume of 1.00 ml. With cultured cell extracts KCN was not needed, as no endogenous NADH oxidation was observed. Phosphate buffer, pH 6.0, was used in all cases except where noted otherwise. All reagents except the buffer were prepared fresh before assay. In some cases the DTT was omitted, as noted under Results. The mixture was equilibrated in the spectrophotometer for about 1 min without NADH; the reaction was then started by the addition of NADH and followed at the wavelength appropriate for the analogue in use. All reaction rates were compared with a water blank and were measured during the interval of 20–80 s after starting the reaction. Enzyme activity was expressed as the change in absorbance (ΔA) per minute per milliliter of enzyme preparation. All assays were performed at 30 °C; constant temperature was maintained with a Lauda circulator. All TH measurements were made in 1-ml cuvettes with a 1-cm light path with the Gilford model 2400 spectrophotometer set to its highest sensitivity: full-scale deflection was set equal to 0.100 absorbance unit. Specific activity was based on protein content as measured by the Lowry method (15), and was expressed as ΔA per minute per milligram protein. Measurements of pH for determinations of optimum conditions were taken directly on reaction mixtures at the end of each analysis with a Beckman Zeromatic pH meter.

Analogues

The analogues used as acceptors in these studies were the 3-acetylpyridine analogue of NAD (3AP-NAD) and the thionicotinamide analogue (TN-NAD). The reduction of 3AP-NAD was followed at 380 nm, and that of TN-NAD was followed at 400 nm, where the contribution to total absorbance by the NADH present was minimal. NADH and the analogues were purchased from the Sigma Chemical Company. Approximate purities were 98% for NADH and 90% for 3AP-NAD; that for TN-NAD was not known.

Preparation of Digitonin Solution

One hundred milligrams of digitonin (Sigma Chemical Co.) were suspended in 1 ml of water and dissolved by the addition of 1 ml of 5 *N* NaOH. Three milliliters of 0.2 *M* potassium phosphate buffer, pH 7.2, were added, and the resulting solution was adjusted to neutrality at the pH meter by the addition of 1 *N* HCl. The final volume was brought to 10 ml with water.

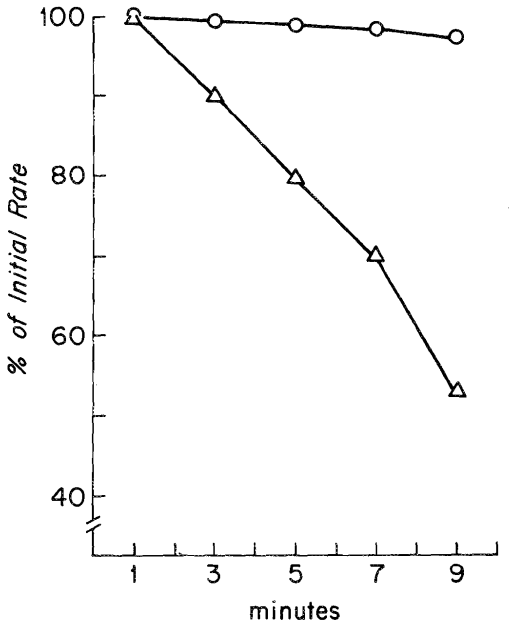

FIG. 1. The effect of dithiothreitol (DTT) on linearity of the transhydrogenase reaction. The protocol for this reaction is given in Methods. The acceptor used was the 3-acetylpyridine analogue of NAD (3AP-NAD) at a level of 1.34 m*M*. (○) Rate of reaction in the presence of 0.15 m*M* DTT during any given 2 min interval over a 9 min period, relative to the rate during the initial 20–80 s; (△) results obtained when DTT was not present in the reaction mixture.

Results

Effects of Dithiothreitol and Digitonin

Transhydrogenase activity was first shown with crude homogenates of H4 cells assayed with tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.6 and with TN-NAD as acceptor. The addition of dithiothreitol (DTT) to the reaction mixture was found to increase significantly the rate of transfer to the analogue.

The dithiothreitol effect was seen also with digitonin-treated cell preparations; this was true with 3AP-NAD as acceptor as well. Fig. 1 illustrates the ability of DTT to sustain the linearity of the rate of transfer of hydrogen to this analogue over an extended period of time. It can be seen that in the absence of DTT the rate of reaction is decreased by 50% in 9–10 min. Not shown here is the ability of DTT to increase the absolute rate of this reaction. This is shown below.

Kaplan *et al.* (1) reported the use of digitonin for the preparation of TH from both bacterial sources and animal tissues. The effects of both digitonin and deoxycholate were tested on H4 preparations. Each of these reagents was found to extract similar units of enzyme activity. Digitonin was used for these studies because it extracted far less protein and resulted in preparations which exhibited a twofold or greater increase in specific activity over that of homogenate supernatants.

Examples of the quantitative effects of digitonin on the specific activity of transhydrogenase prepared from H4 cells are shown in Table 1. It may be seen that complete or near complete recovery of total TH activity in H4 homogenate supernatants occurred when digitonin was used directly in the reaction medium. Note that overnight storage of untreated preparations apparently does not influence the subsequent release of TH activity after digitonin treatment. This release of enzyme activity by digitonin apparently was immediate and could be observed in the spectrophotometer during the reaction. Fig. 2, a replica of an actual tracing on the spectrophotometer, illustrates the kinetics of the digitonin effect. An increase in the rate of reaction is apparent within 1.0–1.5 min after the addition of this reagent to the reaction mixture.

Both digitonin and DTT were used routinely

TABLE 1. Digitonin effect on transhydrogenase activity

Enzyme preparation	Specific activity[a]		Ratio of specific activities[c]
	Immediate	Over night[b]	
1. With digitonin	0.271	0.129	0.48
2. Without digitonin	0.122	0.145	1.19
3. Same as No. 2, but with digitonin added to assay mixture	0.283	0.253	0.90

[a]All assays were performed with DTT present in the assay mixture.
[b]Enzyme preparation was stored for 16–24 h at 5 °C.
[c]Ratio of specific activities: over night / immediate.

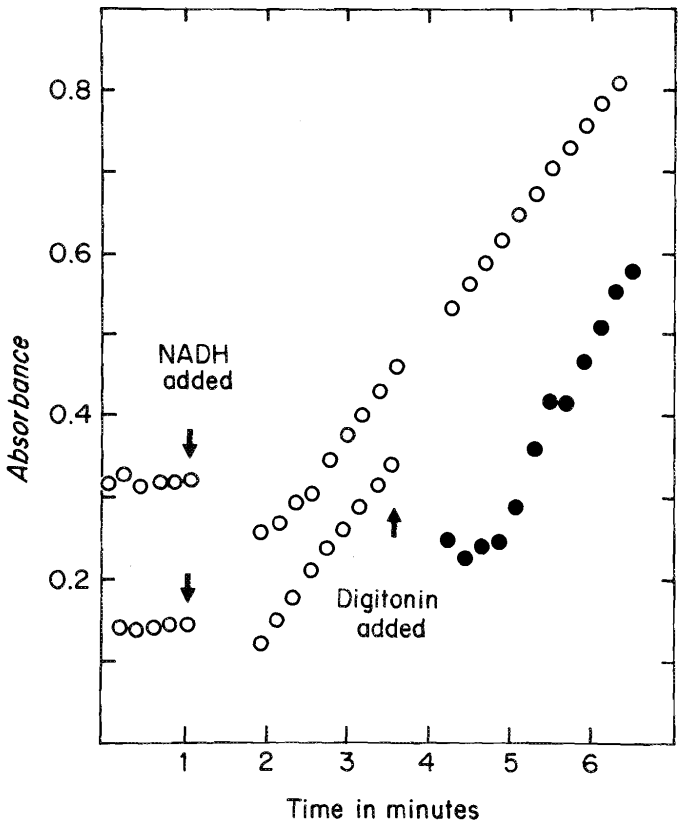

FIG. 2. The immediate extraction of transhydrogenase activity by digitonin. The contents of the initial reaction mixtures are given in Methods. The enzyme used in this case was a supernatant prepared from an H4 homogenate not treated with digitonin. The reaction was started as usual with NADH, as indicated; 0.03 ml of 1% digitonin was added where indicated; an equal amount of phosphate buffer was added to the other cuvette not receiving digitonin. (○) Reaction observed with a preparation never exposed to digitonin; (●) effect of digitonin added after the reaction had begun.

for optimum activity, and most experiments were performed with 3AP-NAD as acceptor. With the protocol described under Methods, typical Michaelis saturation curves were obtained for both 3AP-NAD and NADH with apparent half-saturation values of 3.55×10^{-4} *M* and 9.87×10^{-6} *M*, respectively. Transhydrogenase activity was a linear function of the amount of enzyme preparation used, at least up to an initial ΔA per minute of 0.073.

Lability of Transhydrogenase Preparation

Also shown in Table 1 is the decreased stability of digitonin preparations during over-night storage at 5 °C. Fifty percent or more of the TH activity was lost during this period of time. It was of interest, therefore, to determine the rate of disappearance of transhydrogenase activity in digitonin preparations and to ascertain the cause of the instability of these preparations. Fig. 3 shows the kinetics of disappearance of the ability of cell-free preparations of H4 to transfer hydrogen. Several points are to be noted here. The storage half-life of transhydrogenase activity in digitonin preparations appeared to be around 16–18 h when kept at 5 °C. The very same held true for enzyme preparations in silicone-coated glass test tubes. The half-life for preparations in polycarbonate tubes, however, was much shorter, of the order of only 4 h. This figure also illustrates the stability of supernatants from homogenates not treated with digitonin. Note again that the ability of these latter preparations to respond immediately to digitonin added to the reaction mixture was hardly diminished by 24-h storage at 5 °C.

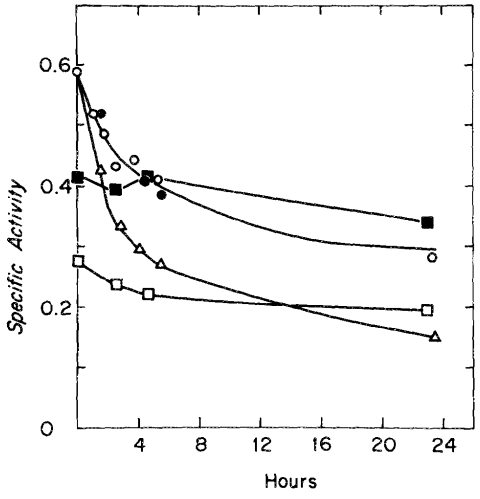

FIG. 3. The stability of transhydrogenase (TH) in digitonin preparations. Supernatants from digitonized homogenates of H4 cells were stored at 5 °C, and TH activity was determined at intervals. The assay method is described in Methods. As acceptor, 1.34 m*M* 3AP-NAD was used, and 0.15 m*M* dithiothreitol was included in all assays. (○) Residual TH activity in preparations stored in glass test tubes; (●) activity after storage in silicone-coated glass tubes; (△) activity of preparations stored in polycarbonate tubes; (□) activity of supernatants from homogenates not treated with digitonin; (■) activity of these latter preparations when 0.03 ml of 1% digitonin was added to the reaction mixture.

pH Optimum: Effect of Tris Buffer

Transhydrogenase activity with 3AP-NAD as acceptor is shown in Fig. 4 as a function of pH and species of buffer used. Several points are worthy of note here. Maximum activity occurred at about pH 5.8. Transhydrogenase was apparently inhibited by Tris; 50–60% inhibition occurred in this buffer over the pH range 7.0–8.5. That this was an effect of Tris and not a pH effect was verified by extending activity determinations in phosphate buffer to pH 8.4. These results are shown in the inset in Fig. 4.

The most interesting effect of DTT is also shown in these figures. This agent usually showed some stimulation of activity in the pH region 5–8. It exhibited its most dramatic influence, however, in reversing the Tris effect. Its action was pH-dependent so that in its presence a second area of optimum activity became apparent between pH 8.0 and 9.0.

A similar profile of activity was observed when TN-NAD was used as acceptor. In this case, a second pH optimum is suggested at pH 7.5. This is illustrated in Fig. 5. The relative effect of DTT on the TH reaction in Tris buffer in the pH region 7.2–8.5 is much greater when this analogue is employed. Again the magnitude of the DTT influence was pH-dependent and showed an optimum effect at about pH 8.0. At this point it increased the rate of reaction about 20-fold over that observed in Tris alone.

Stoichiometry of the Reaction

As an indication of the mechanism of the transfer of hydrogen by H4 preparations, it was desirable to gain some idea of the stoichiometry of the reaction. Theoretical net changes in absorbance expected at any given wavelength can be calculated for hypothetical mixtures of pyridine nucleotides from known spectral properties of individual nucleotides. Further, ratios derived from theoretical changes in absorbance at two wavelengths relate to the stoichiometry of reaction independent of actual changes in concentrations of the reactants. Accordingly, we have calculated ratios of −0.81, −2.68, and −30.40[2] for 2:1, 1:1, and 1:2 molar ratios of NADH and 3AP-NAD, respectively, for net changes in absorbance expected at 380 nm compared with those at 315 nm. Illustrated in Fig. 6 are the kinetics of the TH reaction actually observed at both 380 and 315 nm. The ratio of change in absorbance per unit time at these two wavelengths, $\Delta A_{380}/\Delta A_{315}$, is −2.60 when the higher level of NADH was employed and −3.22 for the lower level. These ratios would correspond most nearly to a 1:1 molar relationship between NADH and 3AP-NAD in this reaction. Accepting a stoichiometric interaction between these two nucleotides, then, we have calculated the actual amount of NADH oxidized per minute by comparing the observed ΔA_{315} to that expected per mole. In the same way we have calculated the amount of 3AP-NAD reduced during the same interval of time from the data obtained at 380 nm. These were 2.38×10^{-3} and 1.34×10^{-3} μmol of NADH oxidized per minute when the higher and lower levels, respectively, were used, and 2.31×10^{-3} and 1.60×10^{-3} μmol

[2]The data published in circular OR-18 (P.L. Biochemicals, Inc., 4th printing, pp. 2 and 3, 1968) were used for these calculations. The data used for 3AP-NAD took into consideration the purity of the preparation. All these values are negative since the absorbance decreases at 315 nm during this reaction.

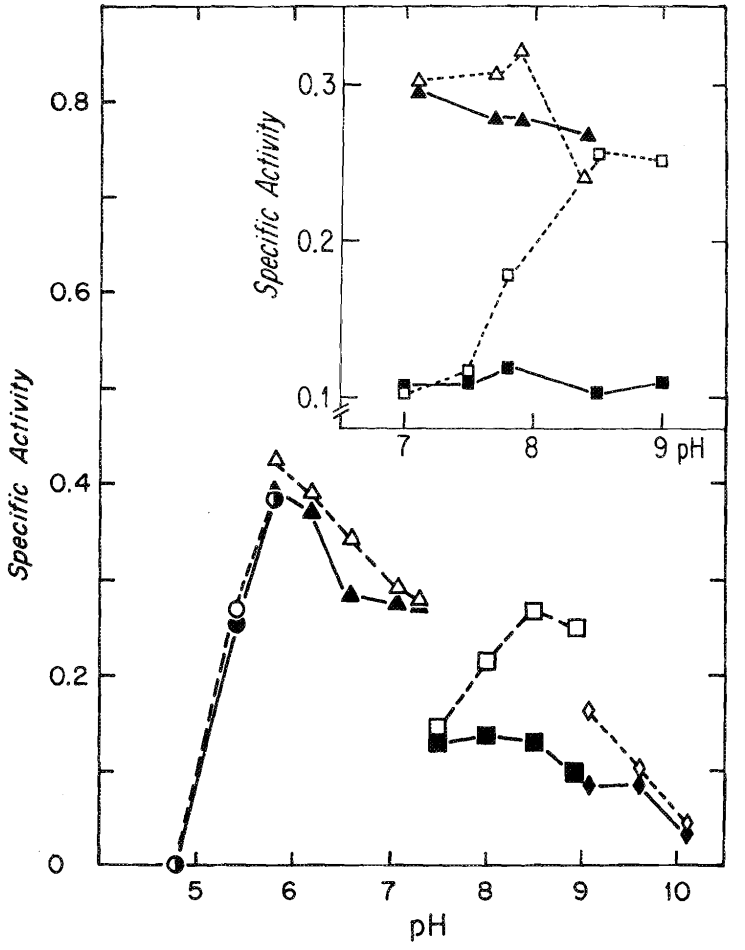

FIG. 4. pH optimum profile for transhydrogenase (TH), with 3-acetylpyridine analogue of NAD as acceptor. Inset: inhibition of transhydrogenase activity by Tris buffer. TH activity was measured at each pH indicated (see protocol in Methods). The various species of buffer used and their symbol designations are: citrate (●), phosphate (▲), Tris (■), and carbonate (◆). The open symbols denote activities observed in the presence of 0.15 m*M* dithiothreitol.

of 3AP-NAD reduced per minute in the corresponding reaction mixtures.

Influence of Various Agents on the Transhydrogenase System

Effects of androsterone, adenosine 5′-triphosphate (ATP), and NAD were tested in this system in two different ways. First, they were added to the reaction mixture after the change in absorbance had reached a constant value. This was to test their ability either to enhance or to reverse the reaction. In order to insure the presence of active transhydrogenase activity, the time necessary to reach constant absorbance was reduced by decreasing the concentration of NADH to one-quarter the usual amount. None of the above agents added at this time could effect either a positive or a negative change in absorbance. The addition of more NADH, however, resulted in a resumption of activity, as indicated by an immediate and continued increase in absorbance. If, on the other hand, androsterone, ATP, or NAD was added before NADH at the start of the reaction, then an inhibitory effect was observed in every case. This is summarized in Table 2. It may be seen that androsterone and ATP inhibit the initial rate of the reaction by about 20 and 30%,

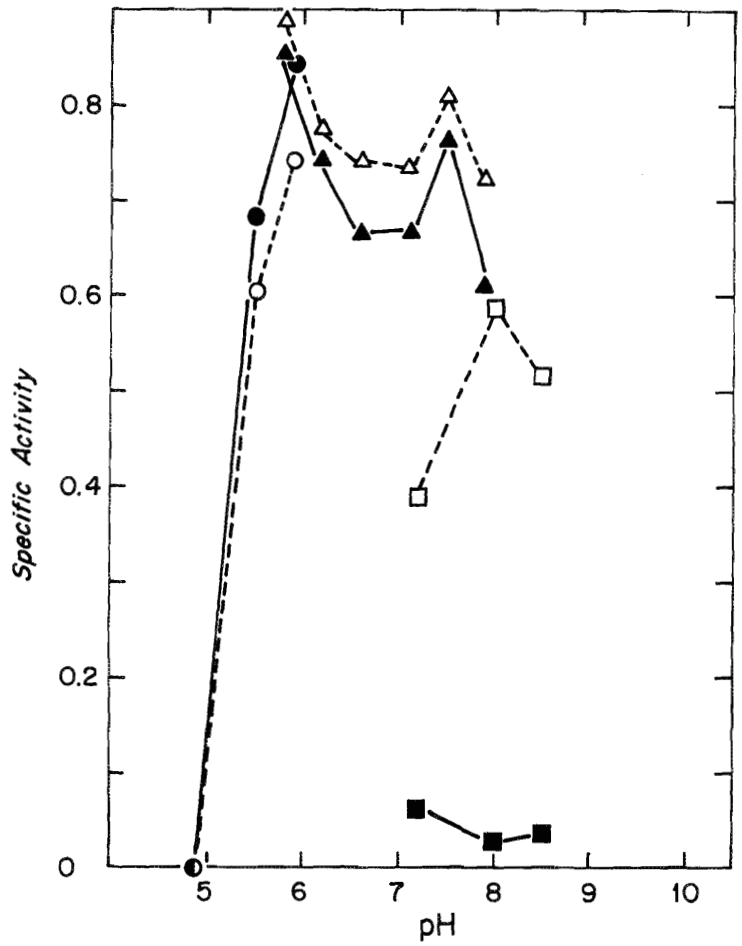

FIG. 5. pH optimum profile for transhydrogenase with thionicotinamide analogue of NAD as acceptor. For legend, see Fig. 4.

respectively. The highest level of NAD used was shown to inhibit the initial rate by about 60% and to lengthen the time necessary to reach a constant absorbance value almost 2.5 times that of a control mixture to which no NAD was added. Androsterone, added with NAD at the beginning of the reaction, apparently did not contribute to the overall inhibition by NAD. The ATP effect, on the other hand, was exerted even in the presence of NAD when they were added together at the beginning of the reaction. Combined, they inhibited the initial rate 72% of the standard and increased the time necessary for the reaction to attain a constant absorbance value by 3.5-fold. Adenosine 2′-phosphate (A2′P), shown by Kaplan *et al.* (16) to influence the reversal of the transhydrogenase system studied in *Pseudomonas fluorescens*, was without effect in this system no matter when it was added. Estradiol, shown by Talalay and Williams-Ashman (7) to mediate transhydrogenase activity in human placenta, could not be kept in soluble form in the system described here. Androsterone, used by Hurlock and Talalay (17) for the liver system, was used instead.

The ability of NADPH to act as donor was also tested with 3AP-NAD as acceptor. It was found that the initial rate of transfer of hydrogen was only 17% that observed in our standard system with NADH.

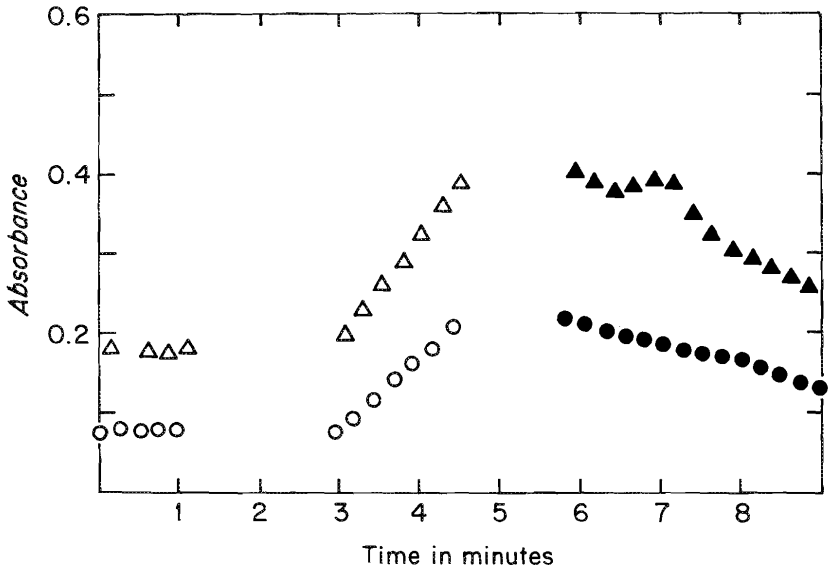

FIG. 6. Stoichiometry of transhydrogenase reaction in preparations from H4 cells. Details of the assay are given in Methods. 3AP-NAD (1.34 m*M*) was the acceptor for these determinations; its rate of reduction was followed at 380 nm. At the break in time seen after 4.5 min, the wavelength selector on the spectrophotometer was changed to 315 nm and the rate of oxidation of NADH was recorded. The open symbols (○, △) denote the reaction observed at 380 nm; the solid symbols (●, ▲) denote that observed at 315 nm. The circles and triangles signify rates obtained with 0.026 and 0.052 μmol of NADH, respectively.

TABLE 2. Effect of various agents on transhydrogenase activity

Addition	Inhibition of initial rate (%)	Relative length of time to reach constant absorbance
None	(00.0)	(1.00)
Androsterone, 1.00×10^{-3} *M*	20.0	1.05
ATP, 8.85×10^{-3} *M*	26.5	1.84
NAD, 0.28×10^{-4} *M*	10.0	1.07
NAD, 1.40×10^{-4} *M*	36.6	1.77
NAD, 2.79×10^{-4} *M*	56.6	2.40
NAD, 2.79×10^{-4} *M* + androsterone, 1.00×10^{-3} *M*	51.5	2.20
NAD, 2.79×10^{-4} *M* + ATP, 8.85×10^{-3} *M*	71.6	3.50

Discussion

Transhydrogenation from one pyridine nucleotide to another has been reported to occur in animal tissues in a variety of ways. The mechanisms proposed for this overall activity encompass direct hydrogen transfer, which may or may not require energy (1, 8–12, 18), for the particulate enzyme to steroid-mediated (6) and steroid-dependent, substrate-coupled (7) reactions of the soluble preparations.

The activity described in this report for cultured mammalian cells would appear to belong to the type first described for animal tissues by Kaplan *et al.* (1). The contribution of digitonin to total activity released and the resulting lability are in accord with this early report. It should be noted that digitonin was not absolutely required to show TH activity in H4 preparations, as was reported by Stein *et al.* (19) for depleted mitochondria from ascites carcinoma. It was required for maximum activity, however. In contrast to the results seen by Kaplan with bile salts, deoxycholate was as effective as digitonin in releasing TH from H4 cells. Dialysis had no effect on

enzyme preparations. The pH optimum observed for TH from H4 cells, pH 5.8, agrees well with that reported by Kaplan and his co-workers for animal tissues. There was no apparent effect of phosphate used as buffer with H4 preparations; this, again, is in agreement with Kaplan's observations on animal tissue preparations, in contrast to those from *Pseudomonas fluorescens* (20). Tris, on the other hand, was inhibitory in this system. Interestingly, DTT was shown to reverse the Tris effect and to do so completely between pH 8.0 and 8.5. The mechanisms for Tris inhibition and its reversal by DTT are not known; they are currently under investigation. The stoichiometry observed appeared to be consistent with a direct transfer of hydrogen from NADH to 3AP-NAD.

The increase in TH activity after digitonin treatment might suggest the possibility of more than one enzyme or more than one form of the same enzyme. The biphasic nature of the stability curves shown in Fig. 3 is consistent with this suggestion. The rapid decline in activity over the first 6 h could be due to a very labile form. The fact that this activity started out at least two-fold higher in the digitonized preparations would argue that the unstable form was that released by digitonin. The activity of the more stable form of the enzyme was manifest after storage for 6 h, when a sharp change occurred in the slope of the decay curve. This was at the same level of activity as that seen with homogenate supernatants, and therefore was entirely consistent with the results obtained with them. The activity of these supernatants would presumably be due to the soluble form of TH; this was seen to be quite stable over 24 h. When digitonin was added at the time of assay, full activity was expressed, that of the soluble plus that solubilized. The fact that the sum of these two did not always reach the level first observed in digitonized preparations can be explained by a loss of particulate enzyme during the first centrifugation of the homogenate. The appearance of a second pH optimum, seen when TNNAD was used as acceptor or when DTT was used to overcome inhibition by Tris, is also consistent with the presence of a second form of the enzyme. Clarification of this point will be possible after further purification of the system showing this activity.

It would appear that the system described here for preparations of H4 cells is not reversible. The lack of influence by NAD added after a constant absorbance value was reached is consistent with this conclusion. The lack of influence of androsterone, ATP, and A2′P, added at this time, points to other major differences from transhydrogenase activities previously described. Their effects, seen when added at the beginning of the reaction, show that they do interact with the system. However, the nature of this interaction is not known and must await further study. The reaction observed upon substitution of NADH by NADPH can be taken to be analogous to the reverse of the standard reaction described in detail in this report. The very weak activity observed in this case cannot be taken by itself as proof of reversibility. In fact, it could well indicate a very stringent, restrictive specificity about the TH that might argue against reversibility. It could, on the other hand, merely indicate the presence of a contaminating enzyme which is not as effective as that utilizing NADH. Clarification of this point must await further purification of the system.

Although the significance of the pyridine nucleotide TH has not yet been completely clarified, a role in regulating relative levels of the oxidized and reduced forms of both NAD and NADP seems most appropriate. The first demonstration of this activity by Colowick *et al.* (21) constituted a classical contribution to the concept and mechanisms of regulatory processes in the living cell. Oldham (22) more recently has emphasized the fact that through TH activity the potential sources of NADP reduction are greatly expanded beyond those available from NADP-linked substrate dehydrogenases. The possible physiologic significance of this enzyme for the H4 cell has been under investigation. Preliminary results of these studies have been published elsewhere (23).

The authors wish to thank Dr. V. R. Potter for his gift of the H4 cell line used in these studies.

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