Fraction 'A' (20–30% of ammonium sulphate saturation) dominates in mouse liver, fraction 'B' (50–60% of ammonium sulphate saturation) dominates in Ehrlich ascites tumor and the muscle cytosolic fractions.
All fractions 'A' were insensitive to L-cysteine. Only fraction 'B' from Ehrlich ascites tumor was markedly inhibited by L-cysteine, but not completely, which also may point to its heterogeneity (table 2).
Among many PK variants two molecular forms were described; the L type from liver and the M type from muscles[12–14]. Neoplastic PK belongs to the type M. It appears, however, that it differs in its sensitivity to L-cysteine. This inhibitory effect does not seem to be caused by the thiol groups, since D-cysteine, or other compounds with free thiol groups such as reduced glutathione or homocysteine, do not influence neoplastic glucose catabolism[7]. It seems thus that this stereospecific inhibitory effect of L-cysteine might be an allosteric effect, characteristic only for tumor PK in a broad spectrum of mouse experimental tumors. Further studies to characterize this PK variant in more detail have been undertaken.

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Recent observations on the structure and the properties of yeast NMN adenylyltransferase

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Summary. A homogeneous preparation of yeast NMN adenylyltransferase (EC 2.7.7.1) showed microheterogeneity, which was revealed by FPLC (Fast Protein Liquid Chromatography) ion exchange chromatography. The resolved components have been characterized with respect to electrophoretic behavior and adenine content. The results led to a hypothesis about a possible role of poly(ADP-ribosylation) in modulating the enzyme activity.
Key words. NMN adenylyltransferase; yeast (*Saccharomyces cerevisiae*); enzyme purification.

Both in the de novo and the salvage synthesis of nicotinamide nucleotides, a central role is played by the nuclear enzyme nicotinic acid/nicotinamide-mononucleotide adenylyltransferase, catalyzing the reaction NaMN (NMN) + ATP → NAD^+ + PPi. The nuclear localization of NAD biosynthesis seems to be associated with the regulation of cellular activities exerted by the nucleus[1]. Indeed, not only NAD biosynthesis, but also NAD degradation is unique to the nuclear compartment, so that the stability of the NAD level in the whole cell appears to be controlled by nuclear events[2]. NAD consumption takes place in the reaction of poly(ADP-ribosylation) of nuclear proteins catalyzed by the nuclear enzyme poly(ADP-ribose) polymerase[3].
The biological significance of this latter reaction is not well understood, as yet; however, data are accumulating suggesting a close relationship between poly(ADP-ribosylation) and many biological events, such as DNA repair, cell cycle, cell differentiation and oncogenesis[4]. The whole of the cell's activity, then, appears to respond to the concomitant fluctuation of NAD levels.
In order to investigate the mechanism of one such phenomenon, we have carried out the purification of the enzyme NMN adenylyltransferase from yeast. Chemical analysis showed the enzyme to be a glycoprotein containing 2% carbohydrates, and two moles of alkali-labile phosphate as well as one mole of adenine derivative per mols of protein (200,000 mol. wt)[5]. Carbohydrate content could be referred mainly to pentoses on the basis of the absorption maximum of the colored product formed, according to the procedure of Dubois et al.[6]. These findings suggested a possible poly(ADP-ribosylation) of the enzyme, that, on the other hand, seems to be plausible in view of the nuclear compartmentation of both NMN adenylyltransferase and poly(ADP-ribose) polymerase. In addition, a modification of this kind seems very likely to possess a regulatory value, both enzymes being involved in the NAD turnover.
However, the data seem only to be consistent with no more than a single ADP-ribose unit per enzyme molecule. The apparent discrepancy might be due to one of the following: a) only a limited fraction of the enzyme molecules is poly-modified; b) the native poly-modified enzymes undergoes extensive demodification (hydrolysis of n-1 ADP-ribose units) at some stage during the purification procedure.
As a result some molecular microheterogeneity would be displayed by the purified enzyme preparation, which might be demonstrated by a sufficiently selective separation proce-

dure. Concomitantly, assuming a regulatory significance of the different degrees of modification, some catalytic microheterogeneity could be shown as well.

In order to recognize poly-modified forms of the enzyme, a new purification procedure was devised, and the features of the final preparation were invesigated. The results obtained are presented in this paper.

Results and discussion. A crude extract was prepared from 250 g (wet) of yeast (*Saccharomyces cerevisiae*) cells by homogenization using a French press, and 3 M KCl extraction, according to the previously reported procedure [5].

In view of the hypothesized demodification of the native enzyme during the purification, and assuming that polyethylenimine (PEI) is a possible demodifying chemical, the PEI step was replaced by a hydrophobic interaction chromatography (HIC) step on pentyl-agarose. The crude extract, equilibrated with 50 mM Tris-HCl buffer, pH 8, containing 25% saturated ammonium sulfate (A.S.), was loaded onto a (5 × 10 cm) pentyl-agarose column equilibrated with the same buffer (Buffer A). The enzyme proved to possess highly hydrophobic properties, binding very tightly to the column. After thorough washing with Buffer A, the column was eluted with a linear gradient of A.S. from 25% to 0% saturation in Buffer A. One single peak of enzyme activity was eluted, between 10% and 5% saturated A.S. concentration, with a smearing up to 0% saturation. Hydrophilic (poly)-nucleotide material was removed with the flow through and washing; substantial removal of contaminating protein was also achieved by this step, resulting in a 50-fold increase of the specific activity. In subsequent steps, represented by DE-52 (step 3) and Green A (step 4) chromatography (table 1), behavior of the enzyme was not different from that in the previously reported procedure [5].

The step 4 material appeared homogeneous when subjected to 5% polyacrylamide disc gel electrophoresis under non-denaturating conditions. One single band was revealed by Coomassie blue staining, whose relative migration (Rm) matched that of the activity assayed on 2-mm slices of a parallel gel. This preparation was further examined for the presence of microheterogeneity. Because of its known resolving power, FPLC was used. Step 4 material, dialyzed against 20 mM Tris-HCl buffer, containing 5 mM 2-mercaptoethanol and 1 mM EDTA, pH 7.5 (Buffer D), was concentrated by ultrafiltration (YM-10, Amicon) and subjected in several aliquots to FPLC chromatography on a Mono-Q HR 5/5 (Pharmacia) column, equilibrated with Buffer D. The elution was performed with a gradient from 0 to 0.5 M KCl in Buffer D.

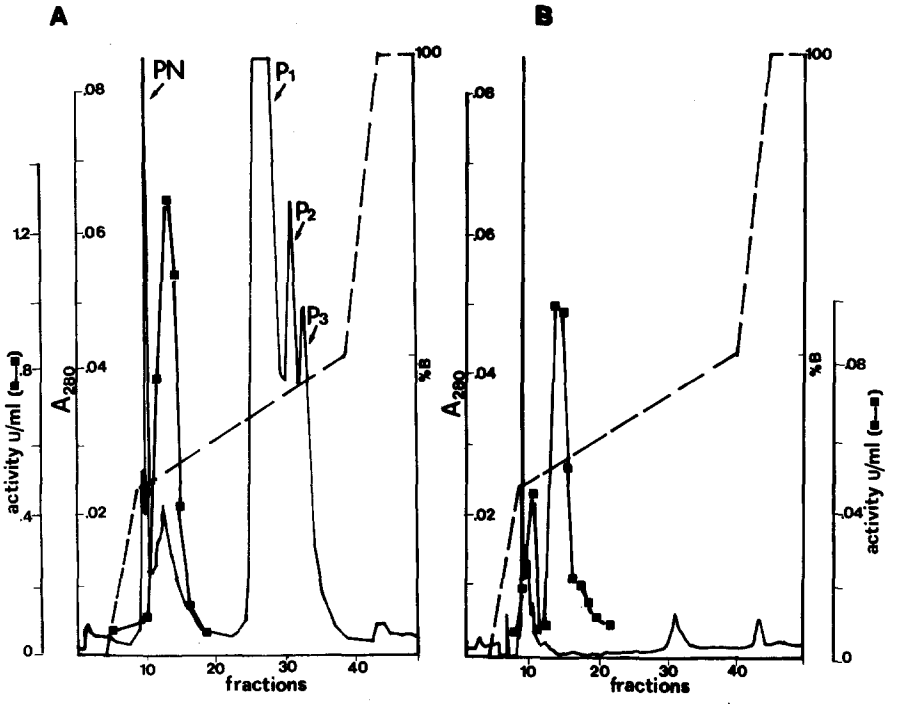
Table 1. Purification of NMN adenylyltransferase

Step	Protein (mg)	Total activity (units)*	Yield (%)	Specific activity (U/mg)	Purification (-fold)
1. Crude extract	12,600	38.8	100	0.003	–
2. Pentyl-agarose	206.7	31	82	0.15	50
3. DE-52	25	15.5	41	0.62	206
4. Green-A	2.3	13.2	35	6.5	2170
5. FPLC Mono-Q	0.6	10	26	16	5330

* One unit of enzyme activity produces 1 µmol of NAD per min.

The enzyme activity eluted between 0.14 and 0.25 M KCl, in a low 280-nm absorbing region (fig., A); in addition, four peaks of 280 nm absorbing material were eluted. The first one (PN) was found to be of nucleotide nature, having an A_{260} over A_{280} ratio considerably higher than 2. The other three peaks (P 1, P 2 and P 3) which were of a protein nature according to their A_{280}/A_{260} ratio, were not well resolved from each other. The fractions exhibiting enzyme activity were pooled, and concentrated (YM-10, Amicon). The final preparation showed a specific activity of 16 U/mg, a purification of over 5000-fold, with a substantial recovery of enzyme activity. A summary of the purification procedure is shown in table 1.

Non-active peaks P 1, P 2 and P 3, which were not well separated from each other, were resolved by further chromatography on the same column by using a different gradient.



FPLC chromatography on Mono-Q HR 5/5 (Pharmacia) column. (——), absorbance at 280 nm; (■–■), enzyme activity; (– – –), gradient profile, from 0 to 0.5 M KCl (0 to 100%B) in 20 mM Tris-HCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, buffer system. *A* Material from step 4 (table 1); pH 7.5 buffer system; *B* Rechromatography of activity peak of (*A*); pH 8.0 buffer system.

Table 2. Incorporation of radioactivity into acid-precipitable material

Reaction mixture*	Incorporation (cpm)	(%)
Nuclei (fresh)	3030	100
Nuclei (fresh) + 3 mM benzamide	320	10
Nuclei (fresh) 20 min incubation	1180	40
Nuclei (thawed)	650	21

* Standard reaction mixture contained: 2 mM [3H] NAD (100,000 cpm); 80 mM Tris-HCl buffer, pH 7.5, containing 8 mM DTT, 3 mM NaF (20 µl); yeast nuclei (40 µg protein, 2 µl); water, up to 125 µl. Reaction was conducted at 25 °C for 5 min and it was terminated upon addition of 125 µl, ice-cold, 20% trichloroacetic acid. After 30 min at 0 °C the acid precipitated material was collected on a Millipore 0.45-µm filter and the radioactivity measured in a liquid scintillation counter.

When analyzed on native 5% disc gel electrophoresis all peaks migrated with the same Rm as the original, confirming the previous findings. However, when run on 5% slab gel, the active peak migrated slightly above the others, whose mobility remained unchanged, and which remained indistinguishable from each other. The native molecular weight was determined by gel filtration on a TSK 3000 FPLC column in the presence of 40 µg/ml of previously purified bovine serum albumin, for the preservation of the enzyme activity [5]. The result showed a single peak of activity eluted as a 200,000-dalton protein. The active peak (fig., A) was also rechromatographed on the same Mono-Q column, using a pH 8.0 buffer system; this allowed the identification of two active peaks as shown in (B) in the figure. The non-symmetrical appearance of the largest peak of activity suggests that it could also be composed of additional unresolved molecular forms.

When purified non-active peaks P 1, P 2 and P 3 were analyzed for adenine derivatives with the glyoxal procedure of Yuki et al. [7] using ADP-ribose as the standard, a value of 3, 3 and 4 moles of adenine derivatives per mole of protein was found for peaks P 1, P 2 and P 3, respectively. This finding allowed us to speculate that P 1, P 2 and P 3 could represent inactive, modified forms of the enzyme, possibly through poly(ADP-ribosylation).

The presence of poly(ADP-ribose) polymerase activity has not been clearly demonstrated in yeast, even though, for such a microorganism, incorporation of radioactivity from labeled NAD into acid-precipitable material has been reported [8]. Our preliminary experiments using yeast extracts failed to demonstrate appreciable incorporation of acid-precipitable radioactivity from NAD. Therefore nuclei were prepared and purified from cultured yeast cells. Yeast nuclei were isolated from exponentially growing yeast cells, with a procedure developed in our laboratory by Dr J. Schwencke, (Laboratoire d'Enzymologie, CNRS, Gif-sur-Yvette, France) (unpubl. results). The purified nuclear preparation was tested for the presence of poly(ADPR)polymerase activity. [3H]NAD (adenine)a was incubated with crude nuclear preparations in the appropriate reaction mixture, and TCA precipitates were tested for incorporated radioactivity. The results showed that appreciable incorporation could only be detected with freshly prepared nuclei, and that prolonged incubation was detrimental to the extent of the incorporation (table 2). The effect exerted by benzamide, a known inhibitor of poly(ADP-ribose) polymerase [3], indicates that incorporation is due to the formation of the polymer. The accumulated evidence suggests that both poly(ADP-ribose) synthesizing enzyme and the synthesized polymer might be subjected to rapid degradation by specific enzyme activities present in yeast nuclei preparations, as already reported [9, 10]. In order to demonstrate unequivocally the modification of NMN adenylyltransferase through ADP-ribosylation, purification of yeast poly(ADP-ribose) polymerase appears then to be necessary. Work is in progress in our laboratory toward this goal.

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Affinity chromatography of glucose dehydrogenase

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Summary. Porcine liver β-D-glucose dehydrogenase, a multi-functional protein, has been purified to apparent homogeneity. The enzyme has been separated from the endoplasmic reticulum using Triton X-114 and further purified using NAD to release glucose dehydrogenase from a NADP-linked sepharose column. The purified enzyme is capable of producing both NADH and NADPH in vivo as indicated by kinetic studies.
Key words. Glucose dehydrogenase; glucose; affinity chromatography.

Glucose dehydrogenase (E.C. 1.1.1.47) is a multi-functional protein [1] capable of binding either NAD or NADP at its active site. This enzyme converts β-D-glucose (NAD) and α-D-glucose-6-phosphate (NADP) to the corresponding cyclic 1,5-lactones while producing either NADH or NADPH for the microsomal electron transport system [2, 3]. Glucose dehydrogenase is located on the luminal side of the endoplasmic reticulum [4] as is glucose-6-phosphatase. Removal of this lipo-protein from the endoplasmic reticulum is readily accomplished with Triton X-114, while its subsequent purification requires four separate chromatographic columns [1, 5].

In this work, we report the successful preparation and use of an affinity column, that takes advantage of glucose dehydro-