On the Problem of Acid-labile Triphosphopyridine Nucleotide in Biological Material

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Recently, Burch et al. (1, 2) reported on the occurrence in tissues of a form of triphosphopyridine nucleotide labile to strong acids. They found on extraction of liver tissue with low concentrations of acid (0.02 M H2SO4 + 0.1 M Na2SO4) an amount of TPN+ which was 4 times higher than that obtained by the usual extraction procedure with high acid concentrations. They concluded that this additional TPN+ was destroyed in the strong acid extraction and that therefore in liver tissue the total content of TPN+ + TPNH is higher, and the ratio of TPNH to TPN+ lower, than reported by earlier investigators (3-6).

Burch, Lowry, and Von Dippe (1) used the common method consisting of extraction of the tissue first and then assay of the pyridine nucleotides. The reduced pyridine nucleotides were measured after alkaline extraction and the oxidized forms after acid extraction. Each extraction must preserve the corresponding oxidation-reduction form of the pyridine nucleotides or else apparent deviations of the total pyridine nucleotide content will be observed.

The extraction method of Burch, Lowry, and Von Dippe (1) was examined with a new enzymatic assay of reduced pyridine nucleotides in alkaline extracts (5) and a method for the assay of TPNH in the alkaline extracts are described in Reference 12.

METHODS

Assay of TPN+ and TPNH in Liver Tissue

Preparation of Extracts—Frozen slices of rat liver (female Wistar II rat, 220 to 250 g, Altrumin fed) were obtained by using the method of Bucher et al. (10).

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For HC104 extraction, 3.0 ml of precooled HC104 (containing 0.6 M HC104 and 5 M ethanol) were added to 150 mg of frozen slices (20 μ thick) at -30°. The sample was then homogenized in a Potter glass homogenizer three times for 90 see each at -5°. After centrifugation and reextraction of the sediment, the supernatant solutions were combined and neutralized with 4 m KOH to pH 3 to 4.

For the weak acid extraction according to Burch et al. (1), 150 mg of frozen liver slices in 3.0 ml of precooled 0.02 x sulfuric acid plus 0.1 M sodium sulfate (pH 2.3) were homogenized with a Potter glass homogenizer for 5 min below 0°. The sample was then heated at 60° for 30 min (applied to the column) and enzymatically assayed without further neutralization.

The preparation of the alkaline extract for the enzymatic assay of TPNH follows the method of Chance et al. (11).

Assay—The enzymatic assays of TPN+ in the acid extracts and of TPNH in the alkaline extracts are described in Reference 12.

The chromatographic assays of extracts from liver tissue were carried out according to Schnitger.1 The columns, filled with Dowex formate-IX8 <400 mesh, were 0.4 mm in diameter and 400 cm in length. The elution was carried out by linear concentration gradient at 21°. The mixing chamber contained 3.92 ml of H2O at the beginning, and the reservoir contained 15.2 m formic acid + 4.8 m ammonium formate. The flow rate was 0.035 ml per hour. Absorbance of the effluent at 265 μm was continuously recorded. For calculations, the molar absorbivity for ADP at 265 μm of 13.3 x 103 μM-1 cm-1 was used.

HC104 extract, 0.123 ml (equivalent to 5 mg of wet tissue) or 0.105 ml of the weak acid extract (equivalent to 5 mg of wet tissue), was applied to the column.

Assay of TPN+ and TPNH in Isolated Liver Mitochondria

Preparation of Extracts—Rat liver mitochondria were isolated according to the usual procedure (cf. Reference 13) from male Wistar rats (Brüning, 200 to 250 g) in a medium containing 0.25 m sucrose, 10 m triethanolamine (pH 7.2), and 1 m EDTA. For reduction of endogenous pyridine nucleotides, the mitochondria (9.6 μg of protein per ml) were incubated aerobically in the presence of 4 μm succinate at 20°.

For HC104 extraction, 1 ml of mitochondrial suspension was added to 0.2 ml of 3 x HC104 and the mixture was allowed to

1 H. Schnitger, unpublished method.
stand at 20° for 20 min. After centrifugation, 0.2 ml of 1 m triethanolamine-HCl was added to the supernatant fluid and the
seed sample neutralized with 3 m KOH to pH 4.

For weak acid extraction, 1 ml of the mitochondrial suspension
was added with good stirring to 2 ml of 0.02 n sulfuric acid in
0.1 m sodium sulfate (pH 2.3) at 20°. The sample was then
cooled to 0° and allowed to stand for 30 min; the pH of the ex-
tract was 2.8. The pH was adjusted to 6 to 7 with 3 m KOH.
After centrifugation, the supernatant solution was subjected to
ion exchange chromatography and to enzymatic assay on TPN.

In a further experiment, the same extraction was carried out
with 0.04 n sulfuric acid in 0.1 m sodium sulfate. The pH of the
extract was 2.3.

In the case of the sample labeled "slowly mixed" (Table II),
the mitochondria were first added to the extraction medium and
the mixture stirred afterwards.

For alkaline extraction, 0.5 ml of 1 N KOH in ethanol was
added with stirring to 1 ml of mitochondrial suspension at 20°,
and the mixture was allowed to stand for 2 hours. Then it
was neutralized by addition of 1 ml of 1 m triethanolamine-HCl.

Assays—Enzymatic assays of TPN+ in the acid extracts were
carried out according to Klingenberg (12).

For the enzymatic assay of TPNH the reoxidation method (7)
was employed. To the neutral supernatant obtained by alkaline
extraction, 10 μl of 0.5 m ketoglutarate, 10 μl of 1 m NH4Cl, and
5 μl of glutamic dehydrogenase (Boehringer) were added to
oxidize TPNH. After 5 min incubation at room temperature,
0.15 ml of 6 N HClO4 was added. After centrifugation, the
supernatant was neutralized to pH 4 and the TPNH originally
present in the alkaline extract was assayed enzymatically as TPN+.

Ion exchange chromatography of mitochondrial extracts was
performed according to Heldt, Klingenberg, and Papenberg (9).
The columns used were 1.1 mm in diameter and 200 cm in length,
filled with Dowex formate-1X8 <400 mesh. The elution was
carried out by a linear concentration gradient at a temperature
2,400 ml per hour. Absorbance of the effluent at 265 nm was continuously recorded.
HClO4 extract, 0.3 ml (equivalent to 2.35 mg of mitochondrial
cell protein) and 0.85 ml of the weak acid extract (equivalent to 3.05
g of protein), was applied to the column.

RESULTS AND DISCUSSION

The occurrence of acid-labile TPN was examined both in whole
liver tissue and in isolated mitochondria. Mitochondria were

Table I

<table>
<thead>
<tr>
<th>Extraction with</th>
<th>Enzymatic assay</th>
<th>Chromatographic assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TPN+</td>
<td>TPNH</td>
</tr>
<tr>
<td>Ethanol-KOH</td>
<td>0.301</td>
<td></td>
</tr>
<tr>
<td>HClO4, 0.5 m</td>
<td>0.063</td>
<td>0.007</td>
</tr>
<tr>
<td>H2SO4, 0.02 N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na2SO4, 0.1 m</td>
<td>0.283</td>
<td>0.276</td>
</tr>
</tbody>
</table>

included, since the chromatographic assay of the reduced and
oxidized pyridine nucleotides (7) and the improved enzymatic
assay for reduced pyridine nucleotides (9) have both been worked
out with this material. Furthermore, the largest part of the
total liver TPN is localized in the mitochondria (6).

Samples from liver tissue and isolated mitochondria were
extracted simultaneously by strong acid, weak acid, and alkaline
ethanol. Tables I and II show the analytical data obtained
from these extracts. The TPN+ and TPNH in the strong acid
extract measured chromatographically agree with the TPN+
measured enzymatically in the strong acid extract and the TPNH
in the alkaline extract. In the weak acid extract, a considerably
higher content of TPN+ is found, which concurs with the report
of Burch et al. (1). This increase of TPN+ is, however,
accompanied by a decrease of the TPNH measured by chromatog-
raphy. Thus, the total content of TPN+ + TPNH amounts to
the same in both extracts.

This shows that the acid-labile TPN+ described by Burch
et al. (1) was not originally present in the sample, but that it was
formed from TPNH during the course of extraction. To further
stimulate the formation of acid-labile TPN, the weak acid was
slowly added to the mitochondrial suspension. Under these
conditions, the amount of TPN found, as shown in Table II,
was even further increased, but again the total content of TPN+
+ TPNH remained unchanged. In another experiment, extrac-
tion of mitochondria was accomplished with 0.04 N H2SO4 plus
0.1 m Na2SO4. Again oxidation of endogenous TPNH was
observed; however, the effect was less pronounced. It is also evi-
dent from Tables I and II that weak acid extraction leads to
extensive breakdown of ATP present in the sample.

From these results it can be concluded that denaturation by
weak acid as introduced by Burch et al. (1) does not give an
immediate quenching of the sample. Thus, artificial transforma-
tion of substances, e.g. oxidation of TPNH and hydrolysis of
ATP, occurs. In view of these findings, the existence of an acid-
labile form of TPN+, as defined by Burch et al. (1), appears to be
highly improbable.

These conclusions are supported by recent findings of Neubert,
Schulz, and Hoehne (14), who observed an oxidation of added
TPNH in weak acid extracts of mitochondria.
SUMMARY

Evidence is given that the acid-labile triphosphopyridine nucleotide, which was reported by Burch, Lowry, and Von Dippe (1) to occur in liver, may be an artifact.

REFERENCES

On the Problem of Acid-labile Triphosphopyridine Nucleotide in Biological Material
Hans W. Heldt, Norbert Greif, Martin Klingenberg, Roland Scholz, Uwe Panten, Joachim Grunst and Theodor Bücher