In human milk, the lactose concentration is about 175 mmol/L, which gives a positive interference as great as 15% with the biuret method (6).

Lönnertal et al. (7) quantified protein in various human milk fractions by four methods (CBB, BCA, Lowry, and Kjeldahl). The CBB and Lowry methods had the lowest variability and both the CBB and the Lowry methods gave protein values closest to those by the Kjeldahl method (7). The BCA method consistently overestimated the protein concentration in human milk by 30% in the above study.

The most nearly accurate method for measuring the protein concentration in milk is the classical Kjeldahl direct analysis (8), which is based on determination of total nitrogen. Before the protein content of human milk is calculated, a correction must be made for nonprotein nitrogen, because the nonprotein nitrogen content is high (15–25%) (7, 9) in human milk. We used the conversion factor 6.25 to convert nitrogen content to protein concentration. The other frequently used conversion factor, 6.38, overestimates the protein concentration of human milk, by not accounting for its high nonprotein nitrogen content.

Ideally, the protein standard should be the same protein that is being determined. In view of the differences in color yield with CBB for different milk proteins (5), human serum is probably the best standard for milk protein assays. We have used a recognized reference human protein standard.

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Interaction between Pyridine Nucleotide Coenzymes and Heme Proteins as a Possible Source of Error in Assay of Activities of Coenzyme-Linked Enzymes

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The ultraviolet absorbance spectra of pyridine nucleotide coenzymes change in the presence of heme-containing proteins. The positions of each of the two main absorbance peaks of NADH are shifted progressively towards shorter wavelengths in the presence of increasing concentrations of hemoglobin, and the third peak, at 220 nm, disappears altogether. Similar effects are seen in the spectra of NAD+ and NADPH, and similar effects on these spectra are produced by myoglobin and cytochrome c, but not by comparable concentrations of albumin. The spectral shifts are generally accompanied by a decreased peak height. This finding may help explain problems reported by previous workers in the measurement of the activity of enzymes such as transketolase or lactate dehydrogenase in erythrocyte hemolysates. Errors may be considerable if allowance is not made for this effect, especially if the concentration of heme protein in the spectrophotometer cuvette much exceeds 1 g/L. The interaction appears to indicate some form of bonding, occurring generally between pyridine nucleotide coenzymes and the heme group in proteins. We relate the findings to measurement of activities of pyridine nucleotide-linked enzymes in erythrocyte lysates and in plasma containing myoglobin after muscle breakdown.

Additional Keyphrases: NADH - NADPH - hemoglobin - myoglobin - transketolase - cytochrome c - erythrocytes - albumin - enzyme activity - variation, source of

Since Negelein and Haas (1) described the measurement of glucose-6-phosphate dehydrogenase activity by measuring the rate of conversion of NADP+ to NADPH, changes in the amplitude of the characteristic absorbance peak for the reduced forms of pyridine nucleotide coenzymes near 340 nm have been used to measure the activities of innumerable enzymes and the concentrations of very many different intermediates of metabolism. Some such measurements have been reported in lysates of erythrocytes, of which one of the commonest is the clinically useful thiamine diphosphate activation test for transketolase (EC 2.2.1.1), often called the "TPP effect," used in the diagnosis of marginal thiamine deficiency (2). This test measures the increase in activity when the cofactor thiamine diphosphate is added to the spectrophotometer cuvette. There are many variations.

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(3) of the basic procedure (4), and technical difficulties have been reported (5). Superoxide dismutase (EC 1.15.1.1), an enzyme believed to play a key role in removing toxic superoxide anion (6), is also commonly measured in erythrocyte hemolysates, although its assay does not depend on changes in pyridine nucleotide coenzymes. During the course of measurements of these two enzymes in human erythrocyte lysates, we encountered problems affecting only the measurement of transketolase, owing to abnormalities in the ultraviolet spectrum of NADH. Here we report our findings on the nature of the anomaly.

Although this particular problem does not seem to have been reported previously, others have also encountered difficulties in work with hemolysates. In the simultaneous determination in biological samples of oxygen consumption and pyridine nucleotide coenzyme redox changes, in which hemoglobin is used as both the oxygen donor and the indicator, errors due to oxygenation-dependent changes in the hemoglobin absorbance must be avoided by using an isosbestic point (7). Other workers have reported suppression of NADH absorbance by hemoglobin (8), potentially a serious problem because the concentration of this protein in erythrocyte lysates is so high. Our starting point, therefore, has been to determine whether the anomalies we have seen in the NADH spectrum are in some way ascribable to the hemoglobin in the hemolysates. We have also investigated the possible occurrence of similar effects between other pyridine nucleotide coenzymes and other proteins.

Materials and Methods

Instrumentation. Absorbances were measured with two different spectrophotometers. We first observed the effect with the DU-7HS scanning spectrophotometer (Beckman Instruments, Inc., Fullerton, CA), but for most of the subsequent work we used an SP800A spectrophotometer (Pye-Unicam, Cambridge, U.K.), a double-beam instrument with linear wavelength scan and a deuterium arc lamp source, in which the beams are alternately switched (25 ms cycle time) onto an end-window photomultiplier. Unless otherwise specified, we used standard quartz cuvettes (10 mm light path), a slit width of 0.3 mm, and external water circulation temperature regulated to 37 ± 0.1 °C.

Reagents. The coenzymes [NAD+ (Grade III), NADH disodium salt (Grade III), NADPH tetrasodium salt], the transketolase substrates (ribose 5-phosphate and xylulose 5-phosphate), the proteins [cytochrome c (Type VI from horse heart) and bovine serum albumin], and the coupling enzymes (glycerol-3-phosphate dehydrogenase and triose phosphate isomerase) were all from Sigma Chemical Co., Poole, Dorset, U.K.

Enzyme assay. We measured in erythrocyte hemolysates superoxide dismutase by a standard procedure (9) and transketolase by the following method.

The transketolase assay medium, prepared in 2-mL batches, consisted of 100 mmol/L Tris buffer (pH 7.59 at 37 °C) containing, per liter, 10 mmol of ribose 5-phosphate, 0.8 mmol of xylulose 5-phosphate, 12 mmol of MgCl2, 1 kU of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), and 0.1 kU of triose phosphate isomerase (EC 5.3.1.1).

To this was added an aliquot, usually 200 μL, of erythrocyte hemolysate sufficient to produce a final hemoglobin concentration of 2 to 2.5 g/L, followed by NADH, 0.1 mmol/L. In the usual transketolase assay procedure, NADH was added last and the subsequent change in spectral absorbance, owing to its conversion to NAD+, was continuously recorded.

Preparation of the hemolysates. Antecubital vein blood (10 mL) was withdrawn from apparently healthy subjects into heparin-containing (20 units/mL) tubes, chilled, and centrifuged (10 min, 1000 × g, 4 °C). As much supernatant fluid, buffy coat, and fat were removed as possible and the cells were washed three times with isotonic saline. The packed cells were frozen (with liquid nitrogen) and thawed three times. We mixed 0.1 mL of this hemolysate with 1.9 mL of Tris hydrochloride buffer (100 mmol/L, pH 7.59 at 37 °C), then stored the hemolysate at −70 °C until used.

Spectrophotometric measurements. We diluted the lysates of human erythrocytes to give a hemoglobin concentration in the cuvette of 1.5 to 2.5 g/L. Besides hemoglobin, the sample cell also contained either NADH (0.14 mmol/L), NAD+ (0.15 mmol/L), or NADPH (0.11 mmol/L). The spectrum of each mixture was scanned from 190 to 450 nm. To identify the cause of the spectral shift, we measured the absorbance spectrum of each pyridine nucleotide coenzyme in the Tris buffer, vs buffer only in the reference cell. The effect of proteins on the spectrum was determined by dissolving them in the buffer to give similar final concentrations (from 0.25 to 3 g/L) of the test protein in both the reference and sample cuvettes. In one set of experiments we repeated these conditions but kept the NADH and heme protein in two separate cuvettes in the same light path, so that no chemical interaction was possible.

Results

The abnormality in the ultraviolet absorbance spectrum of NADH in the transketolase assay medium—a decreased absorbance at the maximum of each peak, and a shift of the wavelengths of the maxima towards shorter wavelengths—could be reproduced by replacing the erythrocyte lysate with the equivalent concentration of hemoglobin only. The effect on the spectrum of NADH of hemoglobin (2 g/L) is shown in Figure 1. The positions of two of the peaks in the absorbance of NADH are shifted towards shorter wavelengths, and their maximum height is decreased. The third peak, which ordinarily displays maximum absorbance around 220 nm, disappeared almost completely, even at the lowest hemoglobin concentration tested, 0.25 g/L. The other changes were dependent upon the hemoglobin con-
centration: as the concentration of the hemoglobin was increased, the wavelength of the maximum absorbance, ordinarily displayed around 260 nm, shifted progressively towards shorter wavelengths, and the absorbance at the maximum was reduced (Figure 2).

Hemoglobin caused similar but rather more complex changes affecting the peak that ordinarily displays a maximum absorbance around 340 nm. It shifted the absorbance peak towards shorter wavelengths, but the peak absorbance increased initially, but not significantly, for hemoglobin concentrations up to about 1 g/L. Then, as the hemoglobin was increased further to 3 g/L, the peak absorbance decreased progressively (Figure 3). The two peaks were affected disproportionately by the hemoglobin, changing the shape of the curve of absorbance vs wavelength for NADH. The effect of a diluted lysate of human erythrocytes on the spectrum of NADH was indistinguishable from that of an equivalent concentration of hemoglobin. The effect of cytochrome c on the spectrum of NADH was indistinguishable from that of a similar concentration of hemoglobin.

When identical hemoglobin solutions were placed in the sample and reference cells without any coenzyme added, the baseline, although noisy, was reasonably flat over the range from 200 to 460 nm. Substantially similar changes in the coenzyme absorption peaks in the presence of hemoglobin were observed with the two different spectrophotometers. When the hemoglobin solution and the NADH were placed in separate cells in the same light path there was no detectable change in the wavelength of the coenzyme absorption peaks, only an increase in noise signals. The changes in the coenzyme absorption only occurred when the solutions were mixed physically. When the hemoglobin concentration (in a separate cell but in the same light path as the NADH solution) was increased to 3 g/L, the apparent absorption of the NADH peaks did alter in a manner quite different to that seen when the hemoglobin and NADH were mixed in the same cell (Figure 1): the tops of the peaks were flattened (Figure 3).

The changes in the coenzyme spectra appeared to be produced specifically by the heme-containing proteins—hemoglobin, cytochrome c, and myoglobin (Figure 4)—but not other proteins. Thus, there was no detectable shift of wavelengths of peak absorbance nor was there any significant change in peak absorbances of the NADH spectrum in the presence of 4 g of human serum albumin per liter. The absorbance of erythrocyte lysate vs buffer did not change greatly in the 300–370 nm range (Figure 5). The shift in the NADH spectrum occurred without any detectable time lag and did not change appreciably over a 6-h period. Decreasing the light path to 5 mm decreased the noise level advantageously but did not affect the spectral shift caused by hemoglobin. The spectrum of NAD⁺ lacks the peak near 340 nm, but the effect of hemoglobin on the other peaks was quite similar to that on the corresponding peaks of the NADH spectrum. High concentrations of hemoglobin changed the spectrum of NADPH in a manner quite similar to their effects on the NADH spectrum.

Discussion

These changes in the ultraviolet absorbance spectra of pyridine nucleotide coenzymes in the presence of heme-containing proteins are of special importance in relation to the spectrophotometric monitoring of enzyme activity by measuring absorbance changes at 340 nm due to redox shifts in pyridine nucleotide coenzymes. It provides an

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Fig. 2. Effect of increasing concentrations of hemoglobin on (left) the shorter wavelength peak and (right) the second peak in the ultraviolet spectral absorbance of NADH
(a) shift in the wavelength of maximum absorbance, (b) change in absorbance at the maximum
Fig. 4. The effect of increasing concentrations of myoglobin (left) on the shorter wavelength peak and (right) on the second peak in the ultraviolet absorbance spectrum of NADH.

- A shift in the wavelength of maximum absorbance, ●—● change in absorbance at the maximum.

Table 1. Effect of Increasing Concentrations of Heme Proteins on the Absorbance of NADH at 340 nm

<table>
<thead>
<tr>
<th>Protein concn, g/L</th>
<th>Hemoglobin</th>
<th>Myoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>105</td>
<td>89</td>
</tr>
<tr>
<td>0.5</td>
<td>102</td>
<td>91</td>
</tr>
<tr>
<td>0.75</td>
<td>114</td>
<td>—</td>
</tr>
<tr>
<td>1.0</td>
<td>102</td>
<td>73</td>
</tr>
<tr>
<td>1.5</td>
<td>87</td>
<td>50</td>
</tr>
<tr>
<td>2.0</td>
<td>44</td>
<td>34</td>
</tr>
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<td>2.5</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>3.0</td>
<td>6</td>
<td>—</td>
</tr>
</tbody>
</table>

Fig. 5. Spectral absorbance of erythrocyte hemolysates, containing around 2 g of hemoglobin per liter, with Tris buffer only in the reference cuvette (5 mm light path).

An explanation for some of the problems reported by previous workers, because erroneous values may be obtained if allowances are not made both for the wavelength shift and the change in peak absorbance of the reduced coenzyme spectrum caused by any heme protein present. In the presence of heme protein it is no longer valid to assume that the molar absorbance of NADH at 340 nm is 6.22 × 10^4, as in the absence of heme proteins. An exception is the case of hemoglobin concentrations not exceeding about 1 g/L, where any error seems to be small (Table 1), apparently because the effect of a small shift in the wavelength is offset by a small increase in absorption (Figure 2). However, as the hemoglobin concentration in the spectrophotometer cuvette is increased above 1 g/L, the two sources of error—the use of a wavelength not giving maximum absorbance and the reduction in overall absorbance around the peak—will both work in the same direction and give an erroneously low estimate of the enzyme activity. Such difficulties have been reported in measurement of the activity of transketolase in erythrocyte lysates. Thus, the observation of Buttery et al. (8), that the NADH absorbance was decreased to 90.5% of the normal value in the presence of approximately 2.3 g/L of hemoglobin, is readily explicable by reference to our data (Table 1, Figure 2). These workers drew attention to the desirability of standardizing the procedures in such a way as to keep the hemoglobin concentration constant. Our findings re-emphasize this warning, and it seems likely that some of the variability in results of other workers in this field is attributable to failure to allow for the complex effects of hemoglobin upon the NADH spectrum. It is clear from the data in Table 1 that the unwarranted assumption that the NADH absorbance at 340 nm is unaffected by heme proteins will cause errors, which become serious at definable protein concentrations—that is, when the protein concentration much exceeds about 1 g/L for hemoglobin or 0.5 g/L for myoglobin. From Figures 1 and 2(right) it is evident also that measurements are better made at a wavelength shorter than 340 nm if the heme protein concentration exceeds about 1.2 g/L, to avoid excessive dependence on the wavelength setting.

The transketolase activation test is believed to provide an indication of thiamine deficiency, because part of the transketolase is in the apo form, and added thiamine
diphosphate is needed to restore activity. Most workers (3,
10, 11) have found that the percentage activation more
reliably indicates thiamine deficiency than does a low
specific activity of the enzyme, and our findings suggest
that the reason for this may be that this relative measure-
ment of activation largely cancels out errors caused by
hemoglobin-induced changes in the NADH spectrum,
which falsify specific activity measurements. It is not
enough just to prepare a standard curve in the presence of
the heme protein. The heme protein concentration expected
in the cuvette should be determined beforehand, the
NADH change measured at the appropriate (shorter) peak
wavelength, and a corrected value for the molar abosrptiv-
ity of NADH used to calculate enzyme activities.

Precautions are needed in the measurement in erythro-
cyte hemolysates of enzyme activities by coenzyme redox
changes—activities of transketolase, alcohol and lactate
dehydrogenases, by reduction of NADH, and activities
of glutathione reductase and glucose-6-phosphate dehydro-
genase, by changes in NADPH. Similar precautions are
needed in the measurement of the activities of blood
plasma enzymes in the presence of myoglobin, generally
released into the circulation after skeletal- and (or) cardi-
ac-muscle injury. The problem can be avoided if the enzyme
can be separated from the heme-containing protein (with-
out appreciable loss of activity) by partition with chloro-
form/ethanol/water, the heme protein being extracted into
the chloroform-rich layer (6).

The wavelength shift towards shorter wavelengths in-
volves both chromophores of the reduced pyridine nucleo-
tide coenzyme. The absorbance peak ordinarily found at
340 nm indicates interaction with the dihydronicotinamide
moiety of the molecule, whereas the other main absorbance
peak, ordinarily around 260 nm, indicates interaction with
the adenine moiety of the NADH molecule. The similarity
of the change in the 260-nm region of NAD$t^+$ absorbance
spectrum in the presence of hemoglobin confirms the se-
parate binding ability of each chromophore (12). Our find-
ings indicate that the interaction is a fairly general one
between pyridine nucleotide and heme groups. Thus, it
affects, on the one hand, both pyridine nucleotide coen-
zymes in both reduced and oxidised forms and, on the other
hand, not only hemoglobin but also at least two other
heme-containing proteins, myoglobin and cytochrome c,
but not a heme-free protein. These findings are consistent
with an interaction between pyridine nucleotide coenzymes
and heme proteins, independent of whether or not the
coenzyme is oxidized. One possibility is that the interaction
is related to the Fe$^{2+}$ in the heme, for the pyridine nucle-
totide coenzymes bind strongly to dehydrogenases (12),
many of which (like heme proteins) contain a tightly bound
divalent cation, usually Zn$^{2+}$. The direction of wavelength
shift indicates that the interaction raises the transition
energy of each of the chromophores in the coenzyme mole-
cule.

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of this work.

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