Direct observation of the NAD glycohydrolase reaction in human erythrocytes using NMR spectroscopy[1]

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Summary. The hydrolysis of NAD by the extracellular membrane-associated enzyme NAD glycohydrolase was shown to be readily followed in concentrated suspensions of human erythrocytes using ^{1}H spin-echo nuclear magnetic resonance spectroscopy (NMR). The maximal rate of the reaction was determined and the inhibitory effect of nicotinamide was confirmed by direct NMR observation. In addition, arginine, ergothioneine and iodoacetate did not influence the reaction rate. ^{31}P NMR analyses of reaction media from whole cells showed that no extraneous degradation of NAD occurred and the only phosphate-containing product was ADP-ribose.

Key words. Human erythrocytes; erythrocytes, human; NAD glycohydrolase; NMR spectroscopy.

Human erythrocyte NAD glycohydrolase (EC 3.2.2.5, NADase) is bound to the external surface of the cell membrane and the hydrolytic cleavage of NAD yields ADP-ribose and nicotinamide. The enzyme is of low activity in human erythrocytes but is high in other mammalian species[2]. The physiological role of the mammalian enzyme is not yet established. However, the A_1 peptide subunit of choleragen[3], *Escherichia coli* enterotoxin[3] and the A peptide of diphtheria[4] toxin have enzymic activities which are of dual specificity; they act as NADases as well as catalyzing an exchange reaction in which ADP-ribose is transferred from NAD to an acceptor moiety. In the case of the *E. coli* and cholera toxins the acceptor is the guanidino group of an arginyl of adenylate cyclase while the diphtheria toxin catalyses the ADP-ribosylation of elongation factor 2 of mammalian protein synthesis[4]; this leads to inhibition of protein production. Turkey erythrocytes possess both the transferase and hydrolase activities but they reside on two separate proteins[5]; this contrasts with human and bovine erythrocytes[6] which appear to have only the hydrolytic activity. In sea urchin eggs a cytosolic enzyme is induced on fertilization and reaches activity levels 50 times those in the unfertilized egg. Again, its role is unknown, however a nuclear enzyme is thought to be involved in DNA repair[7].

A lack of knowledge of the role of this enzyme in normal cellular physiology could, in part, be due to the tedious assay currently available for it[7-9]. This fact coupled with the obvious importance of studies on the bacterial toxins was the motivation for the present work.

The recent discovery of membrane associated phospholipase D in human erythrocytes[10] was made using ^{1}H spin-echo NMR and this suggested that the method could be generally useful for monitoring reactions catalyzed by 'solid-supported' enzymes. An additional advantage of the method is the need for only small cell-sample volumes (~ 0.5 ml). Comparison of the accuracy and precision of the NMR method and that of other methods has been discussed in numerous other papers of biological NMR, and is dealt with in greater detail by York et al.[12] and King et al.[15].

Experimental. Human erythrocytes were prepared from freshly drawn venous blood by centrifugal washing twice in isoosmotic saline at 4 °C and twice in Krebs bicarbonate buffer gassed with carbogen (O_2:CO_2, 19:1)[11] and containing glucose (10 mmol/l). The pH was 7.34 and the buffer contained antibiotics[12]. Hematocrits of cell suspensions were determined by capillary centrifugation[13].

NMR spectra were obtained at 37 °C using a Bruker WM400 spectrometer operating in the pulsed Fourier mode, at 400 MHz for ^{1}H and 162 MHz for ^{31}P nuclei. ^{1}H spectra were obtained by the Hahn spin-echo sequence ($90° - \tau - 180° - \tau$ – acquisition) with τ 0.065 sec[14]. This sequence removes the broad components of the spectrum arising from hemoglobin and erythrocyte membranes. It results in J modulation of the peaks with some of them having negative amplitude. NMR transients (32 per spectrum) were acquired into 8192 data locations with a spectral width of 5 kHz; with repetition time of 2.82 sec per transient. A selective water saturation pulse was applied for 2 s prior to each spin-echo sequence. Chemical shifts were referred to external tetramethyl silane.

^{31}P spectra were obtained with a delay – 90° – acquisition sequence with repetition rate 2.16 sec. Broad band proton decoupling was used with 128 transients averaged in 8192 data locations with spectral width 7 kHz. Chemical shifts were referred to external 85% phosphoric acid.

All fine chemicals were the purest grades from Sigma, St. Louis, Mo., USA. Other chemicals such as salts were of analytical reagent grade.

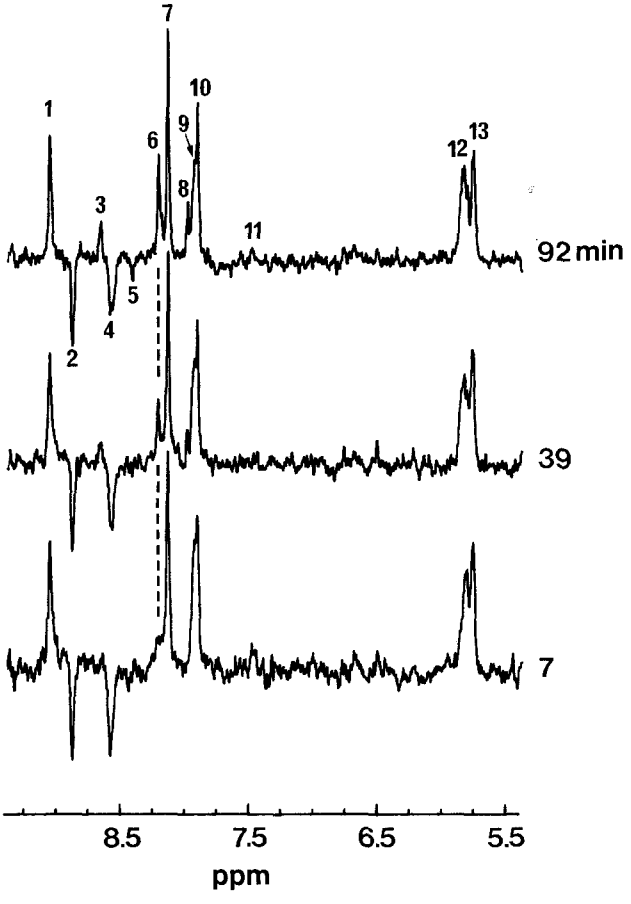

Figure 1. ^{1}H spin-echo NMR spectra of an erythrocyte suspension with added NAD. At t = 0 min, 10 µl of NAD (0.20 mol/l, pH 7.4) was added to 0.5 ml of washed erythrocytes (hematocrit 0.74). Assignments: 1,2,4 NAD-nicotinamidyl H^2, H^6, H^4, respectively: 3,5 nicotinamide H^2 and H^4, respectively: 6,8 ADP-ribose-adeninyl H^8 and H^2, respectively; 7,10 NAD-adeninyl H^8 and H^2, respectively; 9 results from the inverted NAD-nicotinamidyl H^5 resonance superimposed on 10; 11, nicotinamide H^5; 12,13 superimposed ribosyl resonances of NAD and ADP-ribose.

Experientia 41 (1985), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Results and discussion. Figure 1 shows the high frequency portion of a time-series of ^1H spin-echo NMR spectra obtained from a suspension of human erythrocytes; to the sample had been added 10 µl of NAD solution to yield an extracellular concentration of 14.3 mmol/l. The resonance assignments are given in the figure caption; of particular note is the emergence, with time, of free-nicotinamide resonances (3, 8.673 ppm, and 5, 8.427 ppm) and, more prominent, ADP-ribose resonances (6, 8.219 ppm, and 8, 7.985 ppm). The resonance assignments were made by simple addition of buffered solutions of the relevant compounds and noting superposition of peaks in the spectrum. Calculation of the rate of the NADase reaction required concentration-calibration of the resonance amplitudes, which were normalized to the ergothioneine[15] or the glutathioneglutamyl H^{β} peaks (in the low frequency region of the spectrum – not shown). By virtue of superposition of some peaks and the phase modulation of doublets, and other higher order multiplets, not all emerging resonances were equally useful for following the NADase reaction. It is clear from figure 1 that the most prominent and easily measurable peak was that of ADP-ribose-adeninyl H^8 (fig. 1, peak 6). 5-µl aliquots of ADP-ribose (0.2 ml/l, pH 7.4) were added to a sample of erythrocytes (hematocrit 0.74, 0.5 ml) and spin-echo spectra were obtained after each aliquot: the concentrations (mmol/l) and corresponding relative peak amplitudes were: 0.00, 0.00; 3.88, 0.49; 7.55, 1.10; 11.32, 1.56; 14.81, 2.19. A straight line fitted to these data yielded the slope 6.8 ± 0.2 (SD) mmol/l relative peak amplitude units. Thus the direct linear relationship between peak amplitude and concentration was confirmed and the rate of the reaction shown in figure 1 was therefore calculated (after correcting for the hematocrit) to be 2.2 ± 0.2 mmol/l cell·h. An identical value was obtained with cells studied under the same conditions from a different normal blood donor.

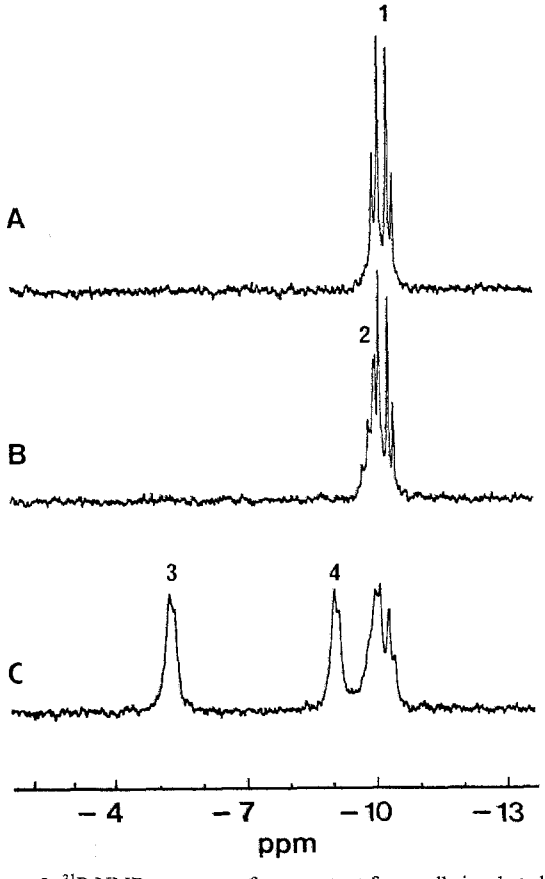

Figure 2. ^{31}P NMR spectrum of supernatant from cells incubated with NAD. At t = 0 min, 0.2 ml of NAD (0.26 mol/l in 0.1 mol/l KH_2PO_4/KOH, pH 7.4, was added to 5.0 ml of washed erythrocytes (hematocrit 0.75). The spectra were acquired from samples prepared at (A) t = 0 and (B) t = 4 h after initiating the reaction. Spectrum (C) was obtained from the sample in B with ADP added prior to spectral accumulation. The spectral assignments are: *A* 1 AB spin-system of the NAD phosphate ester pair; *B* 2 AB spin-system of the ADP-ribose phosphate ester pair; *C* (poorer resolution) peaks common to A and B plus 3 and 4 due to the β and α phosphates of ADP, respectively.

The extracellular NAD concentration used for the experiment, the results of which are shown in figure 1, was greatly in excess of a previously reported value of the Michaelis constant (16 µmol/l)[6]. Also, there has been no reported high-substrate inhibition of the enzyme for the substrate-concentration range used here. Therefore the rate represents the maximal velocity under the present buffer and pH conditions; it is approximately twice the value given in another report[16] on human red cells. The discrepancy is almost certainly due to the different buffer conditions.

Figure 2A shows the ^{31}P NMR spectrum of NAD at pH 7.4 in the supernatant from a red cell suspension, while that in figure 2B shows the effect on the supernatant of incubating the cells with NAD for 4 h. In figure 2B the emergence of the high frequency resonances (2) is consistent with the formation of ADP-ribose, and the absence of any other spectral changes suggested that this was the only phosphate-containing compound, other than NAD, present. If the terminal ribose had been cleaved from ADP-ribose then two resonances, at the chemical shifts of the ADP phosphoryls (3 and 4 in fig. 2C) would have appeared. In addition, if ribose-5-phosphate and AMP had been released as a result of ADP-ribose degradation the corresponding resonances, at higher frequency than inorganic phosphate, would have been obvious.

On the other hand, when ADP-ribose was incubated with a lysate of cells (prepared by sonication[15] of the same cell sample used for the experiment described in the caption of fig. 1) several ^1H spin-echo NMR spectral changes appeared. However, with ADP-ribose added to a suspension of whole cells there were no changes seen in the two purine resonances (H^8 8.219 ppm, and H^2 7.985 ppm) over a 2-h period. In the lysate the most dramatic change was a diminution in the adeninyl H^8 resonance and the elevation of two peaks in close proximity to it, one at a higher frequency (8.309 ppm) and the other at a lower frequency (8.243 ppm). These peaks were readily assigned to ADP and AMP, respectively. After several hours the dominant resonance was that of AMP-adeninyl H^8.

When NAD (3.9 mmol/l final concentration) was added to a lysate the time course of emergence of a resonance in the region of 6 in figure 1 was in fact at a higher frequency and was due to the AMP H^8. This result was consistent with the findings noted in the previous paragraph and quantitative analysis indicated that the NADase reaction operated at the same rate in the hemolysate as it did in a suspension of whole cells. Futhermore, the deribosylation of ADP-ribose was much more rapid than the NADase reaction in the hemolysates. Thus it can be concluded that in *whole* cells insignificant amounts of the ADP-ribose enter the cells.

The arginyl guanidino group is an acceptor of ADP-ribose for NAD in the bacterial transferase reactions[3–5]. Also, it has been suggested that ergothioneine, which has no other ascribed role in red cell metabolism, is an activator of NADase[17]. However, addition of aliquots of either of these compounds, buffered at pH 7.4, to suspensions of erythrocytes with kinetically saturating levels of NAD failed to influence the reaction rate. On the other hand nicotinamide (20 mmol/l) in the suspension medium ensured that no reaction occurred; this is consistent with the previously reported competitive inhibition by this compound[17]. This result also confirmed the effectiveness of high concentrations of nicotinamide to inhibit NAD(PH) break-

down during studies of glycolysis, and other NAD(PH) dependent reactions, in hemolysates[18]. Another reaction which was occasionally, but not reproducibly, seen with NAD in hemolysates (not in whole cell suspensions) was the reduction to NADH. This reaction results in the loss of the aromatic character of the nicotinamidyl moiety and a consequent diminution of the high frequency ^1H spin-echo NMR resonances. The emerging inequivalent geminal H^4 resonances of NADH appeared in the aliphatic region of the spectrum (~ 2.2 ppm). The reaction was inhibited with 3 mmol/l iodoacetate in hemolysates but this compound did *not* alter the NADase reaction rate in suspensions of whole cells.

In conclusion, ^1H spin-echo NMR spectroscopy was established as a valuable means for monitoring the NADase reaction in suspensions of cells (erythrocytes). Various reactions which complicate the interpretation of the results from lysates arise from ADP-ribose degradation, but since the cell is impermeable to this compound the kinetic analysis is simple for whole cells if the adeninyl H^8 resonance is monitored.

1 The work was supported by the Australian National Health and Medical Research Council.

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0014-4754/85/010053-03$1.50 + 0.20/0

Effect of steviol and its structural analogues on glucose production and oxygen uptake in rat renal tubules

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Summary. The effect of several natural products of *Stevia rebaudiana* on glucose production and oxygen uptake in rat renal cortical tubules was investigated. Steviol, isosteviol and glucosilsteviol decreased glucose production and inhibited oxygen uptake. The sweet principle stevioside, and steviolbioside, however, were without effect on gluconeogenesis and oxygen uptake.
Key words. Rat renal tubules; renal tubules, rat; *Stevia rebaudiana;* oxygen uptake, steviol; gluconeogenesis.

Steviol is the aglucone of stevioside (fig. 1), a sweet glucoside abundant in the leaves of *Stevia rebaudiana,* a shrub belonging to the Compositae[1]. Stevioside is 300 times as sweet as sucrose and is now becoming important in Japan and Brazil, mainly as substitute for other non-caloric sweeteners[1]. Aqueous extracts of the leaves of *Stevia rebaudiana,* on the other hand, are used not only for sweetening purposes, but also because they have physiologic and therapeutic effects. It has been claimed that they have cardiotonic[2], contraceptive[3] and hypoglycemic properties[4,5]. Furthermore, it has been demonstrated that stevioside, steviol and other natural products of *Stevia rebaudiana* significantly affect several mitochondrial functions[6,7]. In isolated rat liver mitochondria, oxidative phosphorylation is inhibited by a complex mechanism of action which includes inhibition of adenine nucleotide exchange [6], uncoupling of respiration[7], inhibition of NADH-oxidase and L-glutamate dehydrogenase[6,7], etc. Inhibition of ADP phosphorylation in mitochondria usually has important consequences for the whole organism and the question which now arises is whether stevioside, steviol and related compounds also affect mitochondrial functions in the intact cell. This communication represents our first contribution on this subject. It reports the effect of stevioside, steviol and other natural products of *Stevia rebaudiana* on gluconeogenesis, a biosynthetic route strictly dependent on the available energy in the intact cell. To our knowledge, this is the first report about the effect of *Stevia rebaudiana* natural products on intact cell systems.

Materials and methods. Renal cortical tubules from rats were isolated with collagenase (clostridiopeptidase A; EC 3.4.24.3), essentially as described by Gordon and Hartog[8]. Male albino rats (Wistar strain, 180–250 g) were fasted for 24 h prior to the isolation of cortical tubules. Stevioside was purified from dried *Stevia rebaudiana* leaves as described previously[9]. Steviol was obtained from stevioside by enzymatic hydrolysis with pectinase (poly [1,4-α-D-galacturonide]-glycano-hydrolase; EC 3.2.1.15) as described by Mosettig and Nes[11]. Glucosilsteviol, on the other hand, was obtained by digestion of steviolbioside with the gastric juice of the marine snail *Megalobulimus paranaguensis* (M.L. Ferraresi et al., unpublished). Isosteviol and steviolbioside were prepared from stevioside by strong acid and strong base hydrolysis, respectively[10]. Glucose was assayed enzymatically with hexokinase (EC 2.7.11) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49)[12]. Oxygen uptake was measured polarographically[13]. Protein was determined according to Lowry et al.[14].

Results. The formulae of the five compounds investigated in this work are shown in figure 1. As can be seen, the number of glucose molecules attached to the aglucone moiety decreases