Assay of Flavin Nucleotides in Pancreatic Islets by a Differential Fluorimetric Technique

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Differences in fluorescence intensity between FMN and FAD at high and low pH values were utilized to determine the concentration of each nucleotide. Amounts down to 1 pmol were measurable. The FMN and FAD contents of pancreatic islets of obese-hyperglycaemic mice (gene symbol *ob/ob*) were compared with those of the exocrine pancreas, the liver and heart muscle. As expected, the FAD content of each tissue was greater than that of FMN. The contents of both nucleotides were significantly higher in the islets than in the exocrine part of the pancreas, but lower than in the heart muscle and liver. Possible relationships between the flavin content of the islets and their capacity for oxidative phosphorylation are briefly discussed.

Many specialized cells occur in small groups, as in the nervous system, the nephron of the kidney and the insulin-producing islets of Langerhans, and at present this allows the isolation of only small samples for biochemical studies. The islets are of minute size; commonly a mass of about 1.5 g is distributed among more than one million islets in the human pancreas (Ogilvie, 1937). The islet size distribution is similar, at least in animals used in diabetes research. Their bioenergetic properties have become a matter of considerable interest and determinations of the amounts and interconversion of both adenine nucleotides (Täljedal *et al.*, 1964; Matschinsky & Ellerman, 1968; Hellman *et al.*, 1969; Wettermark *et al.*, 1970; Krzanowski *et al.*, 1971; Borglund, 1973) and nicotinamide nucleotides (Matschinsky & Ellerman, 1968; Brolin *et al.*, 1971, 1972; Berne *et al.*, 1973) have been carried out. Highly sensitive methods meeting these requirements have been developed, permitting measurements of ATP in a single cell (Wettermark *et al.*, 1975). In studies of the mitochondrial function and respiration of the islets it would be of value to measure their contents of flavin nucleotides. This requires the development of current analytical principles into methods with sufficient sensitivity and accuracy for assay of small samples of specialized cells.

Experimental

Experimental animals

For development and tests of the analytical procedure, livers from two male Sprague-Dawley rats were used in addition to the livers from lean littermates of obese-hyperglycaemic mice. Other measurements were carried out on samples of pancreas,

heart muscle and liver from 13 male obese-hyperglycaemic mice (gene symbol *ob/ob*) aged about 6 months. The islets of these mice are comparatively large and in a hyperactive state and thus suitable for our study. The organ samples were removed as soon as possible after induction of anaesthesia with ether.

Chemicals

Trichloroacetic acid (analytical grade), Tris and FAD were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; FMN was a gift from the same company. K_2HPO_4 was obtained from J. T. Barker Chemical Co., Phillipsburg, NJ, U.S.A. The constituents of the Krebs-Ringer phosphate buffer (Krebs & Henseleit, 1932) were obtained from E. Merck, Darmstadt, West Germany, except for $CaCl_2$, which was from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A. Quartz-double-distilled water was used throughout the experiments.

Instrumentation

The measurements on the islet extracts and the other small samples were performed with a Farrand Ratio Fluorimeter at room temperature (about 23°C). The initial methodological experiments were carried out with a Turner Self-Balancing Fluorimeter model 111, with a temperature-stabilized multiple-reflecting cuvette holder mounted on the door (110–655). In all the measurements the filters belonging to the Turner equipment were used. A primary filter 760 was used to isolate the mercury line at 366 nm and the secondary filters 2A and 58 were chosen to give a maximum transmission at about 525 nm. The analyses were based on differences in fluorescence intensities of FMN and FAD at low and high pH

values (Bessey *et al.*, 1949). To meet the specific demands on reproducibility only carefully calibrated pipettes were used. In the methodological investigations a Radiometer pH-meter, type PHM 26c, with a micro-electrode unit, type E 5021, was used.

Preparation of samples

In the initial methodological studies fresh pieces of liver were weighed in small closed vessels and homogenized in double-distilled water with a motor-operated glass homogenizer. After extraction with 12% (w/v) trichloroacetic acid (also resulting in protein precipitation) and centrifugation (10000g, 5 min), samples of the supernatants were taken for assay (Bessey *et al.*, 1949). The islets were isolated by free-hand dissection under a stereomicroscope from fresh pieces of pancreas which had been kept in Krebs-Ringer phosphate buffer at 0–4°C (Hellerström, 1964). After freeze-drying, the islets were weighed on a Lowry quartz-fibre balance with a sensitivity of 154 ng/scale division. Four to six freeze-dried islets, generally representing a dry weight of about 5–10 µg, were inserted into micro-tubes (inner diameter 3 mm) containing 4.9 µl of double-distilled water and frozen in order to disrupt cellular membranes. Homogenization was then carried out with a rotating loop of stainless steel that fitted the tubes well, which were kept immersed in water at 0°C (Eichner, 1966). The speed of rotation was adjustable and the procedure watched through a stereomicroscope. The homogenization was continued for 3–4 min until particles were no longer visible, but was interrupted very briefly for rapid re-freezing, which was repeated when the homogenization was complete. Small freeze-dried pieces of liver, exocrine pancreas (acini) and heart muscle were also treated in the same way as the islets. The micro-homogenates were extracted by adding 23.3 µl of 12% (w/v) trichloroacetic acid. The tubes were then kept on ice for 15 min and protected from light to prevent hydrolysis of FAD.

Analytical procedure

After centrifugation of the trichloroacetic acid/homogenate mixture at 0–4°C, portions (23.3 µl) were pipetted from the supernatant into fluorimeter tubes containing 57.8 µl of 0.2M-K₂HPO₄ and 58.7 µl of double-distilled water, giving a pH between 2.0 and 3.0. After measurement of the fluorescence, the pH was raised to between 6.0 and 7.0 by addition of 3.2 µl of 4M-K₂HPO₄ and a second measurement made. Standards of each nucleotide and blanks were subjected to the entire procedure. In the initial methodological experiments, where larger tissue volumes (liver) were taken, the concentrations of buffers and the relative amounts added were the same. In this series of measurements,

standards of FMN and FAD were also subjected to the entire procedure together with samples and blanks. The standards were thus solutions of the flavin nucleotide in double-distilled water. Since the standard samples were of the same volume as those of the tissues samples, the nucleotide content of the latter could be assessed.

Calculations

Determination of the amounts of FMN and FAD required readings at two pH values, where the subscript 1 denotes the first measurement at the low pH and the subscript 2 the reading at the higher pH. The fluorescence for each nucleotide at each pH value is assumed to be proportional to its concentration. The individual nucleotide concentrations can then be calculated from the ratios between their fluorescence in batches at each pH value. The necessary equations are given in a form suitable for computer calculation as shown below:

$$[\text{FMN}] = \frac{1}{(R_{\text{FMN}} - R_{\text{FAD}})} \times (R_{\text{FMN}}\Delta F_1 - \Delta F_2) \times S_{\text{FMN}}$$

$$[\text{FAD}] = \frac{1}{(R_{\text{FMN}} - R_{\text{FAD}})} \times (\Delta F_2 - R_{\text{FAD}}\Delta F_1) \times S_{\text{FAD}}$$

where R_{FMN} = ratio between the readings for FMN standard (after subtraction of the blank) at high and low pH; R_{FAD} = ratio between the readings for FAD standard (after subtraction of the blank) at high and low pH; $\Delta F_1, \Delta F_2$ = sample fluorescence readings minus blank reading; $S_{\text{FMN}}, S_{\text{FAD}}$ = concentrations of standards. For the statistical evaluation Student's *t* test and analysis of variance were used.

Results

Methodological investigations

The assay used in the present study represents an adaptation to a micro-scale of analytical principles used previously (Burch *et al.*, 1948; Bessey *et al.*, 1949; Burch, 1957). A differential fluorescence assay utilizing a shift of pH has also previously been reported (Faeder & Siegel, 1973). The accuracy of the latter method seems to be quite satisfactory, although the sensitivity attainable does not meet the demands of islet research. Applications on the micro-scale require a detailed evaluation of the various analytical steps, and it is particularly important to ascertain that the mixing of solvents and buffers results in changes of the fluorescence intensity clearly related to the change in pH (Bessey *et al.*, 1949). To check this, equimolar amounts of the two nucleotides were used throughout an experiment in which the pH was raised by addition of portions of K₂HPO₄ at increasing concentration. The results are summarized

Fig. 1. Difference in fluorescence between FAD (○) and FMN (■) at various pH values
Excitation was at 366nm, and readings were taken about 525nm. The amounts of FAD and FMN were kept at 44 and 42 pmol respectively. The rise in pH was obtained by increasing the concentration of K₂HPO₄ from 82 to 205mM, the concentration of trichloroacetic acid being maintained at about 103mM. Fluorescence readings are given as arbitrary units.

in Fig. 1 and show an influence of pH that meets the requirements. A basic requirement for the calculations is proportionality between the nucleotide concentration and intensity of the fluorescence at the pH values concerned. This was checked for small amounts of both nucleotides, and no significant deviations from the required linearity were found (Fig. 2). Low blank readings were obtained in all the experiments. In the initial methodological studies an extraction period four times as long was tested, but did not yield higher amounts of nucleotides. Treatment of FAD standards with trichloroacetic acid did not split off FMN if the time was extended from 15min to 60min. Extraction with trichloroacetic acid for 15min seemed thus to be adequate and free from undesired effects. Experiments in which FAD and FMN standards had been made in liver homogenates instead of double-distilled water showed no interaction with the tissue components. The contents of flavin nucleotides in the liver of Sprague-Dawley rats were 0.061 and 0.019mmol/kg wet wt. for FAD and FMN respectively, which is close to values reported for various strains of rats (Bessey *et al.*, 1949; de Luca *et al.*, 1957).

Assay in hyperglycaemic mice

The larger-scale and micro-scale methods yielded results which were in good accordance as far as the liver was concerned. It seems reasonable therefore to assume that the latter method, which had to be used with the islet tissue, yielded reliable values. The main results are shown in Table 1. Heart muscle had the highest content of both nucleotides, but higher

Fig. 2. Standard curves for FMN (a) and FAD (b) at high and low pH values
Each nucleotide showed satisfactory proportionality between concentrations and fluorescence intensity at both high and low pH values.

Table 1. Content of flavin nucleotides in the pancreatic islets compared with other organs of the obese-hyperglycaemic mouse
All values are expressed as means±S.E.M. and represent μmol/kg dry wt. (duplicate assays). Numbers of animals are given in parentheses.

	FAD	FMN
Heart muscle (10)	291 ± 20	139 ± 9
Liver (13)	241 ± 24	50 ± 10
Islets (13)	139 ± 18	23 ± 4
Acini (13)	113 ± 3	9 ± 2

values of FAD were found in a few samples from the kidney, which is known to possess tubular cells with numerous mitochondria. A statistical evaluation of the material presented in Table 1 showed significantly higher values of FAD than of FMN in all the

different samples. Analyses of variance indicated significant differences in content between each nucleotide. If the FAD values are compared for each animal, e.g. given as a percentage of the liver values, all means show significant differences. This is also found for FMN. The FMN content of the islet is more than twice that of the acini, whereas the FAD content of islets is only 25% higher than in acini. Owing to a smaller distribution, the probability of the latter difference was in the same range, each being statistically well established ($P < 0.005$).

Discussion

In the published method (Faeder & Siegel, 1973) for determinations of FAD and FMN the same principle as in our assay was used and a more detailed account of calculation was given. It was concluded in that previous study that a higher degree of sensitivity could be attained, and this has proved to be true; the tenfold increase in sensitivity reported here will meet the demands of islet research. If measurements below pmol concentrations are desired, a specially built fluorimeter or techniques using bioluminescence seem to be required (Brolin *et al.*, 1971; Stanley, 1971). In fluorimetric analyses of tissues containing appreciable amounts of riboflavin the latter must be removed with a solvent such as benzyl alcohol (Burch *et al.*, 1948). The liver is known to contain small amounts of riboflavin, which was disregarded in our experiments. Certain cytoplasmic enzymes contain a flavin nucleotide as prosthetic group (Weiner & Heppel, 1972). It is conceivable that these nucleotides are mainly associated with mitochondrial function and particularly with oxidative phosphorylation. To what extent the respiratory chain may possess surplus capacity is hardly known for any cells, but the complement of flavins may be adapted to different demands, as exemplified by heart muscle in the present study (Table 1). Whether the values found for the islets imply limitations of their utilization of oxygen cannot yet be settled. Electron-microscopic studies of mitochondria from islets (Volk & Lazarus, 1964; Logothetopoulos, 1966; Wellman *et al.*, 1967) have been carried out for different purposes in several investigations, but so far no form of B-cell mitochondrial dysfunction has been described. Measurements of FAD and FMN represent a biochemical approach by a method which is sensitive enough for further applications in islet research. It has been demonstrated that the entire pool of adenine nucleo-

tides may change in the islets and depends on the supply of glucose (Borglund, 1973). To which extent FMN and FAD are subject to similar changes may be of relevance to the bioenergetics of the islets.

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