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A NEW FLUORIMETRIC METHOD FOR THE DETERMINATION OF PYRIDINE NUCLEOTIDES IN PLANT MATERIAL AND ITS USE IN FOLLOWING CHANGES IN THE PYRIDINE NUCLEOTIDES DURING THE RESPIRATION CLIMACTERIC IN APPLES

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Abstract—A method is described for the determination of the oxidized and reduced forms of pyridine nucleotides* by measuring the increase in fluorescence due to the reduction of resazurin to its highly fluorescent reduced form, resorufin in a recycling system involving a specific dehydrogenase, its substrate, *N*-methyl phenazonium methosulphate and resazurin. The conditions of the assay were arranged so that the level of pyridine nucleotide was rate limiting and under these conditions it was possible to measure amounts of pyridine nucleotides as low as 10 $\mu\mu$mole. Suitable investigations have shown that under the conditions of extraction and assay used, inhibitory effects due to the possible presence of enzyme inhibitors or quenchers of fluorescence in apple fruit extracts are negligible. A study of changes in pyridine nucleotides during the ripening of apple fruits detached from the tree was made. The most marked changes were in NADP and $NADPH_2$ in the pulp, but small but significant changes also occurred in the NAD level both in the peel and pulp. The method of assay was applied to the peel and pulp of pears and to pea leaves in addition to apple tissue and the values obtained are compared with other published values for plant tissues.

INTRODUCTION

IT IS well established that the levels and the relative concentrations of the oxidized and reduced forms of pyridine nucleotides play a fundamental role in the metabolic control mechanism of cells.[1,2] The study of changes in pyridine nucleotides in tissues can therefore provide useful information on the adaption of metabolism to altered physiological conditions. The work to be described in this paper forms part of a continuing study of the regulatory mechanisms involved in the rise in respiration, known as the respiration climacteric, which precedes the ripening of certain fruits including apples and pears.[3,4]

Enzymic methods are now almost always used for the estimation of pyridine nucleotides since they alone provide sufficient specificity to distinguish between chemically similar

* Pyridine nucleotide (PN) has been used as a generic term to include both the oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD and $NADH_2$) and of nicotinamide adenine dinucleotide phosphate (NADP and $NADPH_2$).

[1] F. DICKENS, *Proc. Conf. Enzymes and Their Actions*, Wageningen p. 105 W. E. J. Tjeenk Willink-Zwolle, The Netherlands: N.V. Uitgever-Maatschappij (1959).
[2] J. B. FIELD, I. PASTAN, B. HERRING and P. JOHNSON, *Biochim. Biophys. Acta.* **50**, 513 (1961).
[3] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. London, Ser. B.* **158**, 514 (1963).
[4] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1343 (1967).

nucleotides. In general, the methods for the estimation of pyridine nucleotides fall into two categories:

(1) Those methods involving the measurement of the accumulation of an end product in which the amount of product formed is equal to the amount of cofactor being measured.[5, 6]

(2) Those methods in which the nucleotide undergoes a series of repeated oxidations and reductions leading to accumulation of the end product in amounts considerably greater than that of the original pyridine nucleotide.[7–14]

Owing to the low levels of pyridine nucleotides in most tissues, the latter methods, (recycling methods), which clearly have considerable advantages in terms of sensitivity, have been most widely used. They are, however, more prone to interference by inhibitory substances in the extracts being studied. In general with recycling methods, the recycling of the nucleotide is linked on the one hand to the action of a specific dehydrogenase enzyme and on the other, via a suitable electron mediator, to the production of the end product. The level of pyridine nucleotide is estimated from the rate of production of this end product.

Fluorimetric methods are amongst the most sensitive available for the estimation of a wide range of biologically important compounds.[15] Lowry *et al.*[8] have developed extremely sensitive fluorimetric methods for the estimation of pyridine nucleotides in animal tissues based on a complicated recycling system. This involves a final direct fluorimetric measurement of the reduced pyridine nucleotide, in which the excitation wavelength is 365 nm and the fluorescence is measured at 470 nm. Both acid and alkaline apple extracts fluoresce strongly in this region making direct measurements of reduced pyridine nucleotides difficult. Quenching of fluorescence is also troublesome in this region. Another fluorimetric method for the estimation of nucleotides is that of Jacobson and Astrachan,[5] but this is of the non-recycling type.

Guilbault and Kramer[16] recently described the use of the dye resazurin in the measurement of the activity of dehydrogenases. The oxidized dye, resazurin, is non-fluorescent, but on reduction is converted to resorufin which is highly fluorescent at 580 nm when excited at 560 nm. At these wavelengths apple extracts do not fluoresce. This paper describes an enzymic recycling method based on the fluorimetric measurement of the reduction of resazurin.

RESULTS

Fluorescence Characteristics of Apple Extracts

Figure 1 shows fluorscence emission spectra of a neutralized dilute acid extract of apple peel, chlorogenic acid and $NADH_2$. When the extract is excited with light of wavelength 350 nm, there is a maximum of fluorescence at 460 nm which closely corresponds with the

[5] K. B. JACOBSON and L. ASTRACHAN, *Arch. Bioch. Biophys.* **71**, 69 (1957).
[6] I. PASTAN, V. WILLS and B. HERRING, *J. Biol. Chem.* **238**, 3362 (1963).
[7] G. E. GLOCK and P. MCLEAN, *Biochem. J.* **61**, 381 (1955).
[8] O. LOWRY, J. PASSONNEAU, D. SCHULZ and M. ROCK, *J. Biol. Chem.* **236**, 2746 (1961).
[9] T. F. SLATER and B. SAWYER, *Nature* **193**, 454 (1962).
[10] Y. YAMAMOTA, *Plant Physiol.* **38**, 45 (1963).
[11] A. GREENBAUM, J. CLARK and P. MCLEAN, *Biochem. J.* **95**, 161 (1965).
[12] G. S. SERIF and F. R. BUTCHER, *Anal. Biochem.* **15**, 278 (1966).
[13] G. S. SERIF, L. SCHMOTZER and F. R. BUTCHER, *Anal. Biochem.* **17**, 125 (1966).
[14] D. GRAHAM and J. E. COOPER, *Australian J. Biol. Sci.* **20**, 319 (1967).
[15] S. UDENFRIEND, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York (1962).
[16] G. G. GUILBAULT and D. N. KRAMER, *Anal. Chem.* **37**, 1219 (1965).

fluorescence maximum of $NADH_2$ (see Fig. 1). This is not due to $NADH_2$ but to interferring substances, probably phenolics, (see spectrum of chlorogenic acid). When the extract is excited at 546 nm, there is no fluorescence at 580 nm corresponding with the intense fluorescence maximum of resorufin.

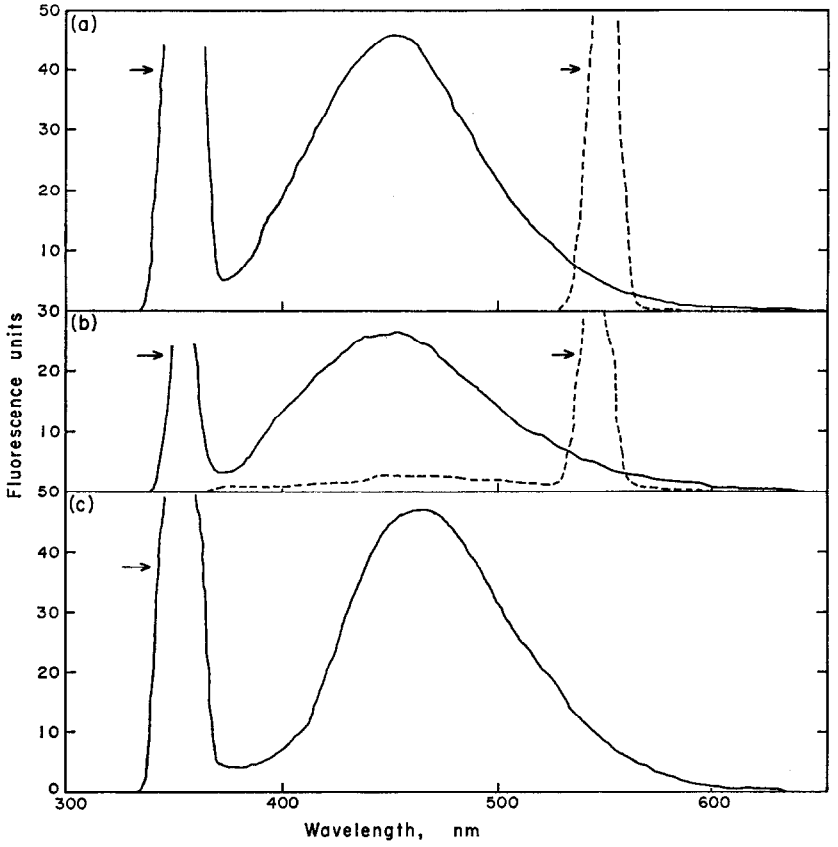

FIG. 1. THE FLUORESCENCE EMISSION SPECTRUM OF (A) A DILUTE ACID EXTRACT OF APPLE PEEL PREPARED IN THE STANDARD MANNER (SEE EXPERIMENTAL SECTION) (B) CHLOROGENIC ACID IN WATER AND (C) $NADH_2$ IN WATER.
The continuous lines show spectra excited at 350 nm and the discontinuous lines excited at 546 nm. The peaks at 350 nm (continuous line) and 546 nm (discontinuous line) shown by an arrow represent scattering (not fluorescence) due to the primary exciting beam.

The Assay System for Pyridine Nucleotides

(*a*) *General.* The recycling systems used for the estimation of pyridine nucleotides in apple tissue is illustrated in Fig. 2. The same systems were used for the estimation of both the oxidized and reduced form of each nucleotide. The oxidized forms were extracted from the tissue in acid extracts under conditions in which the reduced forms were destroyed, and conversely the reduced forms were extracted under alkaline conditions which destroy the oxidized forms. An enzyme specific for the particular nucleotide under study was coupled by means of *N*-methyl phenazonium methosulphate (PMS) with the reduction of the dye, resazurin, to its highly fluorescent reduced form, resorufin. Alcohol dehydrogenase (ADH)

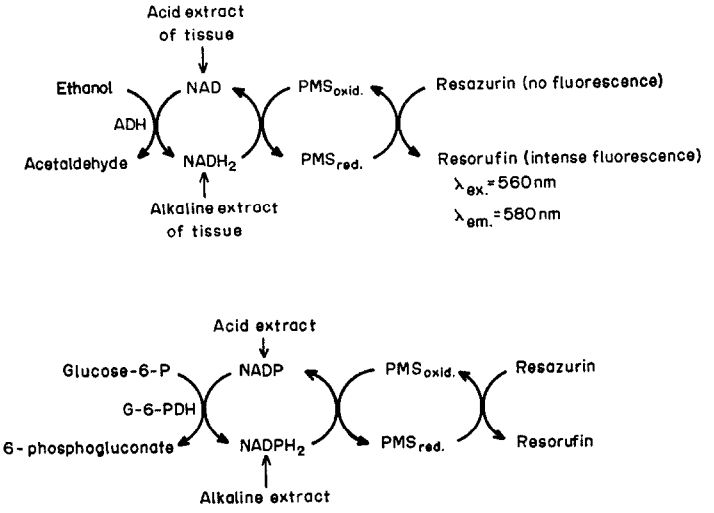

FIG. 2. DIAGRAMMATIC REPRESENTATIONS OF THE SYSTEM USED FOR THE ESTIMATION OF NICOTINAMIDE ADENINE DINUCLEOTIDES (UPPER DIAGRAM) AND OF NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATES (LOWER DIAGRAM).

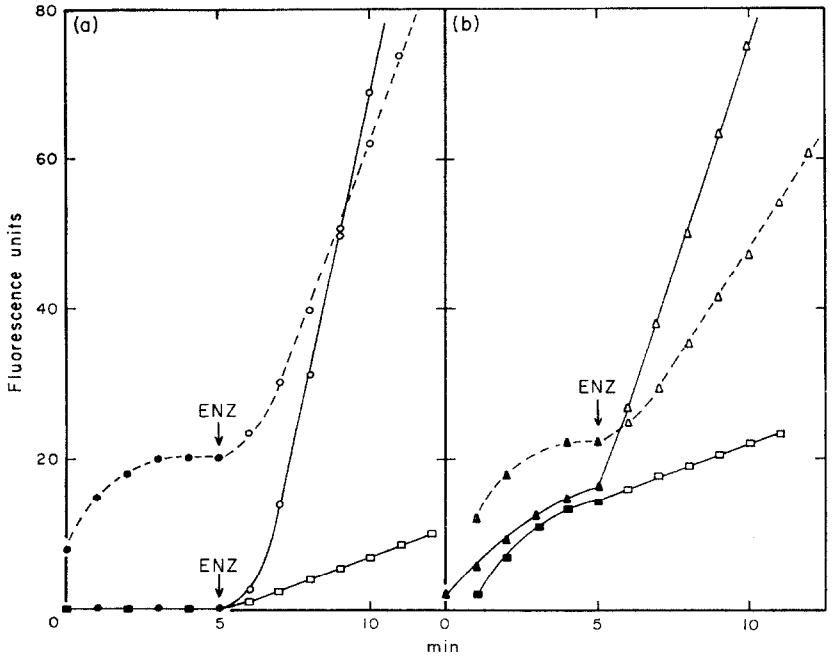

FIG. 3. TYPICAL ASSAYS OF EXTRACTS AND STANDARD SOLUTIONS OF AUTHENTIC NUCLEOTIDES FOR (A) NAD AND (B) NADP. THE CURVES SHOWN ARE REPRODUCED FROM THE RECORDER TRACINGS OF INDIVIDUAL ASSAYS.

In 3A the curves shown represent the assay of distilled water blank (□——□) 172 $\mu\mu$mole NAD (○——○) and 0·05 ml of an acid extract of apple peel prepared in the standard manner (○----○). Each assay was allowed to proceed for 5 min (solid symbols) prior to the addition of the enzyme ADH (see arrow).

In Fig. 3B the curves shown represent the assay of a distilled water blank (□——□), 72 $\mu\mu$mole NADP (△——△) and 0·2 ml dilute acid extract of peel tissue (△----△). As in Fig. 3A assays before the addition of enzyme are shown with solid symbols.

and glucose-6-phosphate dehydrogenase (G-6-P DH) were used respectively for the estimation of NAD and NADP. The concentration of pyridine nucleotide in the assay system is proportional to the rate of dye reduction which was measured as an increase in fluorescence in the system. In all the assays, all the reactants except enzyme were mixed and changes in

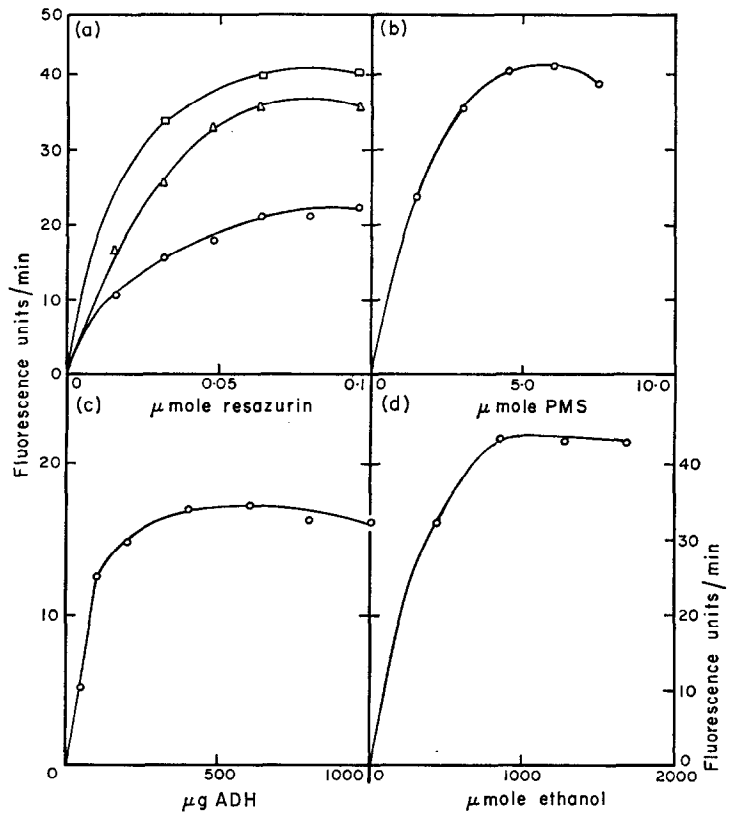

FIG. 4. THE EFFECT OF VARYING THE CONCENTRATION OF VARIOUS COMPONENTS OF THE ASSAY MIXTURE ON THE RATE OF DYE REDUCTION IN THE ASSAY OF 150 μμMOLE OF NAD.

4A The effect of varying the resazurin concentration at three concentrations of PMS: (○——○) 1·5 μmole, (△——△) 3·0 μmole (□——□) 4·5 μmole PMS.

4B The effect of varying the concentration of PMS at a concentration of 0·1 μmole Resazurin.

4C The effect of varying the concentration of alcohol dehydrogenase in the assay.

4D The effect of varying the alcohol level in the assay.

fluorescence followed for about 5 min, the enzyme was then added and, after a lag of about 2 min, the fluorescence increased linearly with time for about 10 min (see below). The calibration curve for the assay was shown to be a straight line for each of the four nucleotides up to a concentration of about 200–250 μμmole (see Fig. 5). Above this value, the rates of reaction are linear only for about 4 min, and then fall off rapidly. The lower limit of the sensitivity of the method is approximately 10 μμmole, which gives rates which are about twice the blank value.

In the assay of extracts, it was found that non-enzymic reduction of the dye occurred before addition of the enzyme (Fig. 3). This is probably due to the presence of reducing

substances in the extract. In general, this problem is greater with acid than with alkaline extracts. However, in all cases the rates of dye reduction fall off almost completely by the end of 5 min. Therefore the incubation mixture without enzyme was followed until the rate of increase in fluorescence fell to zero. Non-enzymic reduction becomes more complicated in the NADP assay due to the effect of EDTA which increases the degree of non-enzymic reduction, even in the assay of a standard solution of NADP. This reduction falls with time but never falls below 1·0 unit per min. There is no further increase in this rate upon addition of enzyme in a blank assay. Typical assays are illustrated in Fig. 3. In the assay of extracts

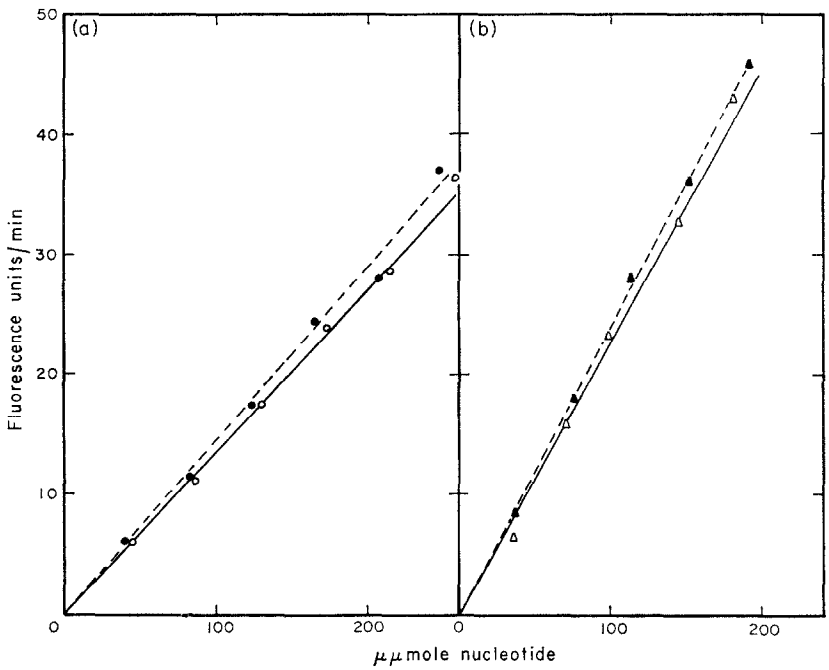

FIG. 5. CALIBRATION CURVES OF RATE OF DYE REDUCTION MEASURED AS RATE OF INCREASE IN FLUORESCENCE IN THE SYSTEM AGAINST PYRIDINE NUCLEOTIDE CONCENTRATION.
NAD (○), $NADH_2$ (●), NADP (△), $NADPH_2$ (▲).

the amounts taken were arranged to give rates of reduction between 5–30 fluorescence units/minute, corresponding to approximately 20–200 $\mu\mu$mole of pyridine nucleotides in a 3 ml reaction mixture.

(*b*) *Factors affecting the Assay of* NAD *and* $NADH_2$. Figure 4 shows the effect of varying the concentration of various components of the reaction mixture in the assay of a standard solution of NAD. The rate of reaction for a given concentration of nucleotide is dependent upon the levels of both PMS and resazurin at low concentrations (see Fig. 4 A and B). At higher concentrations, i.e. 4·5 μmole PMS and 0·1 μmole resazurin per 3 ml reaction mixture, the rate is independent of the concentrations of these two reactants. However, at such concentrations (above 3·3 μmole/3 ml assay—see Ref. 11) PMS undergoes photo-oxidation and can act as a potential electron donor; in our assay system high levels of PMS lead to high and steadily increasing blank rates (approximately 7 units/min). It is possible to alter the range of linearity of the method by varying the PMS concentration.[11] To extend the range

to lower pyridine nucleotide levels it is necessary to increase the concentration of PMS. In much of the present work we have worked at non-saturating levels of PMS (1·5 μmole/3 ml assay) which gave sufficient sensitivity for estimating the levels of pyridine nucleotides found in our tissues, and low blank values (approximately 1·0 unit/min). When working with non-saturating levels of PMS it is essential to prepare calibration curves for each nucleotide daily. Under these conditions, the levels of enzyme (600 μg) and ethanol (850 μmole) used

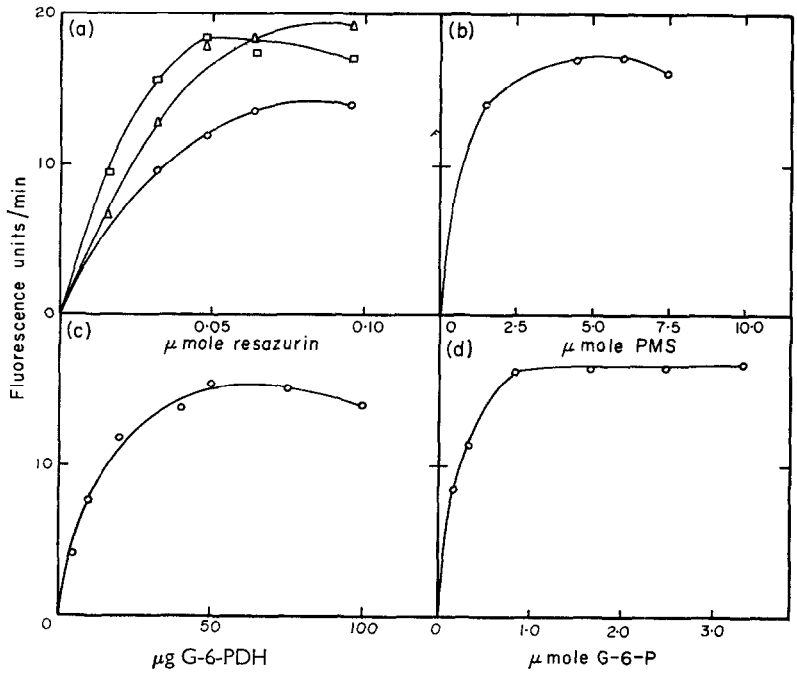

FIG. 6. THE EFFECT OF VARYING THE VARIOUS COMPONENTS IN THE SYSTEM USED FOR THE ASSAY OF 65 μμMOLES NADP.

6A The effect of varying the resazurin concentration at various levels of PMS: (○——○) 1·5 μmole, (△——△) 3·0 μmole, (□——□) 4·5 μmole.

6B The effect of varying the PMS concentration at a concentration of resazurin of 0·1 μmole/assay.

6C The effect of varying the glucose-6-phosphate dehydrogenase concentration in the assay.

6D The effect of varying the glucose-6-phosphate concentration in the assay.

in the assay are in saturating concentrations (see Figs. 4C and 4D). A typical calibration curve is shown in Fig. 5 for both NAD and $NADH_2$ and is linear between 0–250 μμmole/assay.

(*c*) *Factors affecting the Assay of* NADP *and* $NADPH_2$. With the NADP assay again 4·5 μmole of PMS and 0·1 μmole of resazurin per 3 ml assay completely saturate the system but for the reasons given above we have generally worked at non-saturating levels of PMS (i.e. 1·5 μmole/3 ml assay) and, under these conditions, the enzyme level (50 μg glucose-6-phosphate dehydrogenase) and the substrate level (1·65 μmole glucose-6-phosphate) used are sufficient to saturate the system. These results are shown in Figs. 6A–D. Typical calibration curves for NADP and $NADPH_2$ are shown in Fig. 5.

The effect of addition of EDTA to the NADP assay system is shown in Table 1. Although EDTA introduces the need for a small blank correction, it leads to a three-fold increase in the rate of dye reduction. In spite of the disadvantage of the blank correction, EDTA at a concentration of 1·5 μmole/3 ml assay was used in all assays. Table 1 also shows that EDTA has very little effect in the NAD assay either on the blank rate or on the enzyme dependent rate of dye reduction.

TABLE 1. EFFECT OF EDTA ON THE ASSAY OF NAD AND NADP

μmole EDTA added to assay	Rate of dye reduction (fluorescence units/min)			
	NAD μμmole		NADP μμmole	
	0	200	0	76
0	1·5	31·0	0	6·5
0·03	—	—	—	19·0
0·3	—	—	—	19·0
1·5	1·5	31·5	1·0	18·0

The Extraction and Estimation of Pyridine Nucleotides from Fruit Tissue

(*a*) *Extraction and Determination of the oxidized forms.* The methods used for the extraction of pyridine nucleotides are based on those employed by Glock and McLean.[7] In the extraction of the oxidized forms of pyridine nucleotides, various methods have been compared (see Table 2). Extraction with either 0·7 N perchloric acid (PCA) or 0·1 N hydrochloric acid in the cold leads to incomplete extraction of both NAD and NADP (see Table 2—Experiment 1). Heating the 0·1 N HCl extract at 60° for 1–5 minutes leads to further release of pyridine nucleotides. It was subsequently found for both peel and pulp tissue that two extractions with 25 ml of 0·1 N HCl heated at 60° for 4 min, together with exhaustive washing of the residue, was sufficient to give quantitative extraction from 5 g of tissue (see Table 2—Experiment 1). Table 2 also shows that the use of 0·02 N sulphuric acid—0·1 M sodium sulphate, as recommended by Burch *et al.*[17] for the extraction of NADP from liver, did not lead to an increase in the level of NADP found in our apple tissue as compared with the standard hydrochloric acid extraction.

Figure 7 shows the rate of dye reduction in the assay system with different amounts of acid extract for the oxidized pyridine nucleotides. With both NAD and NADP, the graphs are linear and pass through the origin after correction for the blank rate in the absence of extract.

Owing to the possible presence of inhibitors of the assay system (or quenchers of fluorescence—see later) in the tissue extracts it has been necessary with each extract to test for such inhibition. This has been done by estimating a given level of standard and extract separately and together in the same assay and comparing the expected with the observed rate in the combined assay. Table 3 illustrates the freedom from such inhibition of the acid extracts of both peel and pulp tissue of the apple (see also Table 4). Table 3 also shows the result of

[17] H. B. BURCH, O. H. LOWRY and P. VON DIPPE, *J. Biol. Chem.* **238**, 2838 (1963).

TABLE 2. COMPARISON OF EXTRACTION METHODS FOR OXIDIZED PYRIDINE NUCLEOTIDES
μg PYRIDINE NUCLEOTIDE/10g FRESH WEIGHT

Extractant	Conditions of extraction	NAD		NADP	
	Experiment 1. *Peel and pulp tissue*				
		Peel		Peel	
0·7 N Perchloric Acid	0–2°	97		11	
0·1 N HCl	0–2°	78		4·5	
0·1 N HCl	1 min 60°	128		7	
0·1 N HCl	2 min 60°	186		23	
0·1 N HCl	5 min 60°	178		18	
0·1 N HCl	0–2°	64		1	
	re-extracted 1 min 60°	127		8	
0·1 N HCl	3 min 60°	168		12·5	
	re-extracted 5 min 60°	36		5·0	
		Peel	Pulp	Peel	Pulp
0·1 N HCl	4 min 60° + 4 min 60° + Wash	231	43·6	26	7·4
	re-extracted 4 min 60°	0	0	0	0
	Experiment 2. *Peel tissue*				
0·1 N HCl	Present paper experimental section	224		35·0	
0·02 N H_2SO_4 –0·1 N Na_2SO_4	Burch *et al.*[17]	222		34·5	

recovery experiments in which authentic oxidized pyridine nucleotides were added to the tissue during the extraction procedure. The Table shows that good recoveries were obtained with both NAD (98·5–105 per cent) and NADP (97–103 per cent) within the range investigated.

It was found that the oxidized nucleotides in an acid tissue extract were destroyed if the extract was made alkaline. This extract was re-neutralized and used to provide a blank for the assay of the extracts. This blank value was equal to that given in the absence of extract.

(*b*) *The Extraction and Estimation of the Reduced Forms.* For apple tissue, the method of Glock and McLean[7] for the extraction of the reduced pyridine nucleotides has been modified by the use of 0·1 N potassium hydroxide in 50 per cent ethanol instead of in aqueous solution. The presence of the ethanol helps to precipitate pectic compounds during extraction and leads to clear extracts without the necessity of using high speed centrifugation. As with the acid extracts it was necessary to use heat to give complete extraction. Three successive treatments at 60° for 4 min were required to give complete extraction of the reduced forms from the tissue. In all the assays for $NADH_2$ the alcohol content of the assay was adjusted to a constant value with due allowance for the presence of ethanol in the tissue extract. Although ethanol is not required as a substrate in the determination of $NADPH_2$, since ethanol is present in the extracting medium, in the final assay the ethanol concentration was always adjusted to a constant level. This level of ethanol was shown not to be inhibitory in any way.

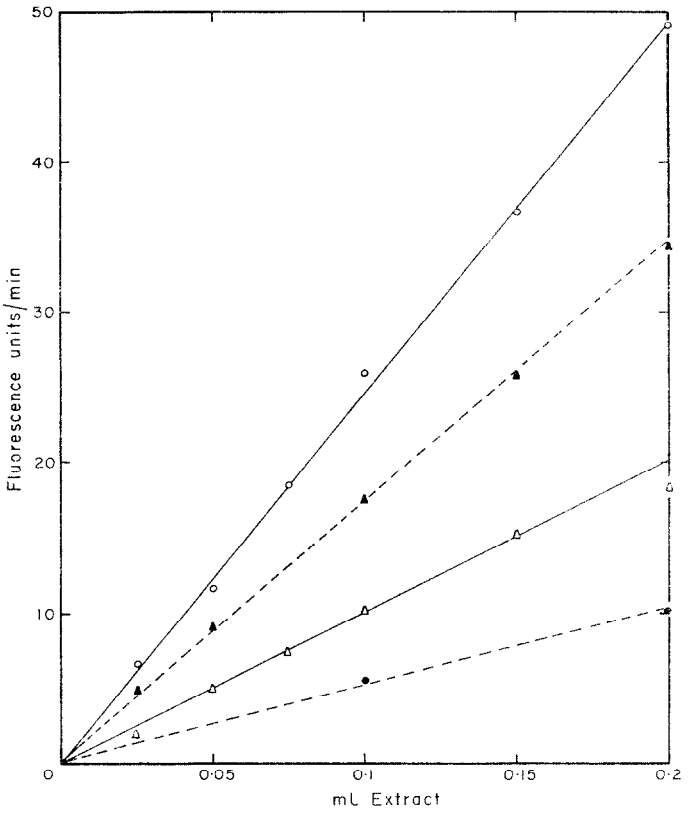

FIG. 7. THE EFFECT OF VARYING THE VOLUME OF TISSUE EXTRACT IN AN ASSAY ON THE RATE OF DYE REDUCTION.

The lines shown represent the following: (○——○) a standard acid extract of apple peel assayed for NAD; (●----●) a standard alkaline extract of apple peel assayed for $NADH_2$; (△——△) a standard acid extract of apple peel assayed for NADP; (▲----▲) a standard alkaline extract of apple peel assayed for $NADPH_2$.

As with the oxidized forms, the amount of extract in an assay mixture for either $NADH_2$ or $NADPH_2$ has a linear relationship with the rate of dye reduction (see Fig. 7) and the line passes through the origin. Table 3 illustrates the inhibition test (see Experimental Section) applied to the alkaline extracts and recovery experiments in which authentic nucleotides were added to the tissue during extraction. In the $NADH_2$ assay the extract is essentially free from inhibition and good recoveries of added $NADH_2$ (96–100 per cent) were obtained. The small activation (up to 10 per cent) in the inhibition test in this experiment with $NADPH_2$ is not usual and the per cent recovery in the inhibition test has nearly always approached 100 per cent (range 93–105 per cent) (see Table 4). The recoveries of $NADPH_2$ added during extraction were also close to 100 per cent.

Blank values for alkaline extracts in which the extracts were made acid to destroy the reduced nucleotides, re-neutralized and then assayed, were the same as the blank value in the absence of extract.

TABLE 3. THE RECOVERY OF AUTHENTIC PYRIDINE NUCLEOTIDES ADDED TO APPLE TISSUE PRIOR TO EXTRACTION

	μg/75 ml	% Activity in inhibition test	μg PN added /75 ml ext.	% recovery
	Peel			
Determination of NAD				
1	76·5	94	25	98·5
2	75·0	92	50	102·0
3	66·0	99	75	101·0
4	73·5	104	100	105·0
Determination of NADP				
1	11·0	93	25	101·0
2	10·5	91	50	97·0
3	13·0	—	75	101·0
4	10·5	98	100	103·0
Determination of $NADH_2$				
1	10·1	103	25	96·0
2	10·5	99	50	99·0
3	13·3	108	75	104·0
4	11·2	96	100	102·0
Determination of $NADPH_2$				
1	35·2	108	25	103·0
2	32·8	108	50	110·0
3	34·5	114	75	99·0
4	31·5	112	100	108·0
	Pulp			
NAD	21·8	110	50	96·0
NADP	3·7	113	50	98·8
$NADH_2$	12·0	90	50	95·0
$NADPH_2$	12·0	102	50	104·0

The Use of Phenolic Binding Agents in the Extraction of Pyridine Nucleotides

Two phenol binding agents, polyclar AT and polyamide (Woelm) have been added to extracts in order to reduce the levels of phenolics. Both polyclar AT and polyamide reduce the level of phenolics in the extracts as estimated by a modification of the Folin–Denis method[21] (see Table 4), more especially with the acid extracts, but have no effect on the level of pyridine nucleotides or on their freedom from inhibition as judged in the inhibition test.

Change in Pyridine Nucleotides during the Ripening of Apple Fruits

Figure 8 shows changes in the levels of pyridine nucleotides in both peel and pulp tissue during the ripening of apple fruits detached from the tree. The fruit was picked in the pre-climacteric state and was then stored at 12° for up to 43 days. Respiration measurements (see Fig. 8) were made daily[3] and samples were taken at intervals for analysis as the respiration climacteric developed.

Figure 8 shows that although the levels of pyridine nucleotides are considerably higher in the peel of the apple compared with pulp, in both tissues the nicotinamide adenine dinucleotide is mainly present in the oxidized form while nicotinamide adenine dinucleotide phosphate is mainly in the reduced form.

TABLE 4. EFFECT OF PHENOL BINDING AGENTS ON THE LEVELS OF PYRIDINE NUCLEOTIDES AND PHENOLIC COMPOUNDS IN APPLE EXTRACTS

	Acid extract					Alkaline extract				
Phenol binding agent	NAD μg/10 g fresh wt.	% recovery in inhibition test	NADP μg/10 g fresh wt.	% recovery in inhibition test	Total phenolics mg/10 g fresh wt.	$NADH_2$ μg/10 g fresh wt.	% recovery in inhibition test	$NADPH_2$ μg/10 g fresh wt.	% recovery in inhibition test	Total phenolics mg/10 g fresh wt.
Control	126	109	14·0	94	38·4	35	101	81	99	23
+Polyclar AT	135	98	16·0	95	17·2	33	101	77	101	17
+Polyamide	132	106	16·6	99	17·4	30	106	81	99	20

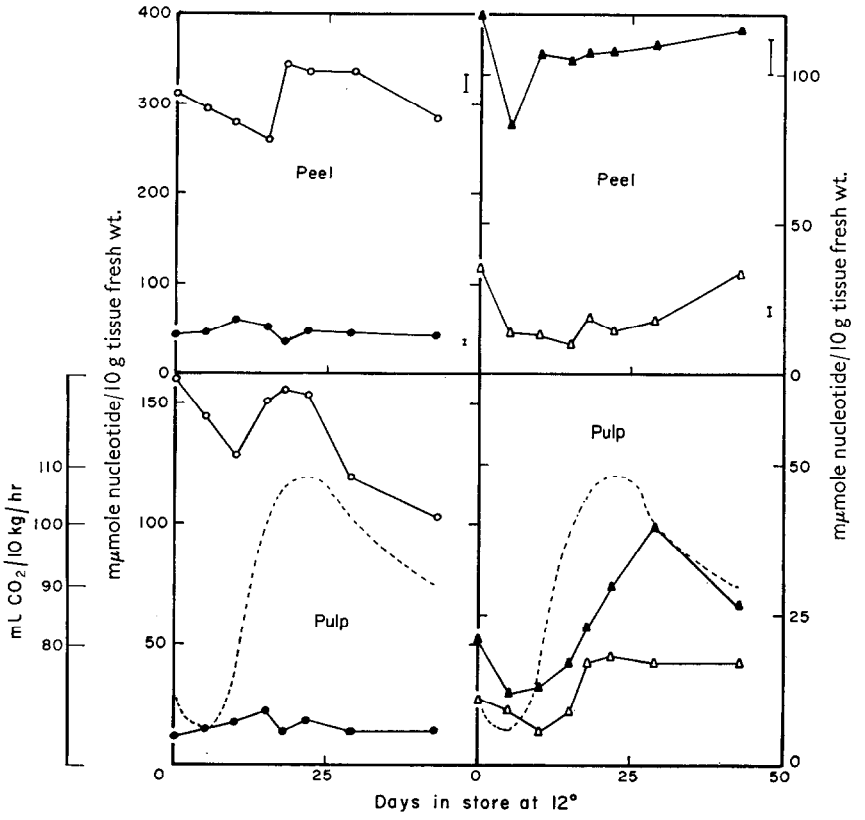

FIG. 8. CHANGES IN THE LEVELS OF NAD (○——○), $NADH_2$ (●----●), NADP (△——△), AND $NADPH_2$ (▲——▲) IN BOTH PEEL (UPPER PAIR OF GRAPHS) AND PULP (LOWER PAIR OF GRAPHS) TISSUE OF APPLES STORED FOR VARIOUS PERIODS OF TIME AT 12°C. IN THE TWO LOWER CURVES THE BROKEN LINES SHOW THE CO_2 PRODUCTION OF THE WHOLE APPLE FRUITS AND INDICATE THE DEVELOPMENT OF THE RESPIRATION CLIMACTERIC.
The least Significant Differences (P=0·05) for the determination of each pyridine nucleotide in peel tissue is shown as a vertical line to the right of the appropriate graph.

The ratio of $NADH_2$/NAD is similar in both the peel and the pulp but the $NADPH_2$/NADP ratio is considerably higher in the peel than the pulp, mainly due to the higher levels of $NADPH_2$ in the peel. The concentration of NAD rises to a peak in both the peel and the pulp and this corresponds with the climacteric peak. However there are no corresponding changes in $NADH_2$. The rise in NAD level is small in quantitative terms compared with the rise in respiration of the whole fruit. There are no consistent changes in NADP or $NADPH_2$ in the peel but there is a two fold increase in NADP and a four fold increase in $NADPH_2$ in the pulp just after the climacteric peak.

DISCUSSION

Enzymic recycling systems of the type used here are open to interference by inhibitors present in the extract and as fruit tissues contain relatively high concentrations of phenolic compounds, especially in the peel tissue, it was necessary to establish that such interference was

Table 5. The estimation of pyridine nucleotides in various plant tissues

		mμmole PN/10 g fresh wt.						
		NAD	$NADH_2$	$\frac{NADH_2}{NAD}$	NADP	$NADPH_2$	$\frac{NADPH_2}{NADP}$	Reference
Apple (Cox's Orange Pippin)	Peel*	222±6	21±1·7	0·10	22±0·9	61±3·8	2·8	Present work
	Pulp	100	10	0·10	10·0	11·0	1·0	Present work
Pear (Williams' Bon Chrétien)	Peel	434	74·3	0·17	50	130	2·6	Present work
	Pulp	120	15·4	0·13	41	61·2	1·5	Present work
Pea leaves (*Pisum sativum*)† (actively growing green leaves)		463	50·2	0·11	389	241	0·62	Present work
Pea leaves		177	29	0·16	94	9·4	0·10	11
		128	—	—	101	—	—	20
Bean leaf (mμmole PN/μmole chlorophyll)		28	1	0·04	21	9·0	0·43	18
Tobacco leaf (young) mμmole PN/leaf		0·38	0	0	3·96	1·12	0·28	10
(old)		11·46	0	0	49·5	3·84	0·07	10

* Mean of 6 determinations performed on a sample of gas-stored Cox's Orange Pippin apples together with the standard deviation.
† Mean of 3 determinations.

not quantitatively significant under the conditions used. The interference could be due either to the presence in the tissue extracts of general or specific inhibitors of the enzymes used (i.e. alcohol dehydrogenase or glucose-6-phosphate dehydrogenase) or to the presence of quenchers of fluorescence.[15] Owing to the sensitivity of the present method it has been possible to assay the pyridine nucleotides in small volumes of relatively dilute tissue extracts and this in addition to the presence of large excesses of the enzymes used in this assay has done much to eliminate interference by enzyme inhibitors present in the fruits. The results show in fact that both interference due to quenching and to enzyme inhibitors have been successfully overcome for the tissues used. It is, however, essential in applying methods of this type to other tissues to establish that the extracts are free from enzyme inhibitors or quenchers of fluorescence.

Table 5 shows a comparison of some of our data for apple and pear fruits and for pea leaves with other published values for plant tissues. The levels of NAD and $NADH_2$ in apple peel are considerably less than those in pear peel and actively growing pea leaves. The levels of NADP and $NADPH_2$ in apple peel are half those in pear peel but very much less than the levels in pea leaves. All the values for pea leaves obtained in the present work are considerably higher than those quoted by other workers.[11,20] It is however difficult to make strict comparison between leaves which probably differ in age and physiological state. The ratio of $NADPH_2$/NADP was greater than unity in both the peel and the pulp of both apples and pears (i.e. 1·1–2·8). This differs from the ratio in pea leaves (see also values for pea, bean and tobacco leaves quoted in Table 5) in which the ratio is less than unity (0·62). This low $NADPH_2$/NADP ratio appears to be related to the photosynthetic activity of green tissues[18] and it is interesting to note that the green peel of fruit tissues differs from leaves in this respect. A high $NADPH_2$/NADP ratio has also been found in most animal tissues that have been studied.[11]

The small but significant rise in NAD which occurs as the respiration climacteric develops in detached apples stored at 12° is small relative to the increase in respiration of the whole fruit. From our data it appears very unlikely that the onset of the climacteric is limited by the availability of pyridine nucleotides. The increases in both NADP and $NADPH_2$ which occur only in the pulp are proportionately greater than the rise in NAD. The physiological significance of these changes in nucleotide levels is as yet unclear.

EXPERIMENTAL

Materials

Fruit was taken from 16 Cox's Orange Pippin apple trees on Malling IX rootstocks at the Burlingham Horticultural Station, Norfolk. The apples were picked in the preclimacteric state and allowed to ripen at a constant temperature of 12°C. Daily respiration measurements were made by the method described by Hulme *et al.*[3] and samples taken for analysis at intervals as the climacteric developed.

For many experiments during the development of the assay system gas or cold stored apples were obtained from Messrs. Norfolk Fruit Growers. Pears were purchased in the local market. Pea leaves were harvested from 3-weeks-old seedlings (var. Feltham First) growing in the light (9000 lux) at 18°.

Extraction of Oxidized Pyridine Nucleotides

Thin strips of peel or pulp tissue were cut with a potato peeler and dropped immediately into liquid nitrogen. The frozen tissue was ground to a fine powder in liquid nitrogen in a pestle and mortar. Five g of this powder was weighed into a centrifuge tube which had previously been chilled with liquid nitrogen. Polyamide

[18] W. L. OGREN and D. W. KROGMANN, *J. Biol. Chem.* **240**, 4603 (1965).
[19] O. H. LOWRY, J. V. PASSONNEAU and M. K. ROCK, *J. Biol. Chem.* **236**, 2756 (1961).
[20] D. G. ANDERSEN and B. VENNESLAND, *J. Biol. Chem.* **207**, 613 (1954).
[21] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agri.* **10**, 64 (1959).

(250 mg, Woelm for thin layer chromatography) was added and mixed in with the powder, after which 25 ml of ice cold 0·1 N HCl was added. The acidified powder was then stirred continuously with a glass rod at 60° for exactly 4 minutes. The extract was centrifuged at 2800 *g* for 10 min. The residue was re-extracted with 25 ml 0·1 N HCl for 4 min at 60°, washed with a further 5 ml 0·1 N HCl and the various extracts combined in another tube containing 100 mg polyamide standing in ice. The combined extracts were adjusted to pH 6·8 with 5 M K_2CO_3 in the presence of polyamide. The precipitate which formed was centrifuged off and washed with cold distilled water. The combined supernatant liquids and washings were made to 75 ml with distilled water. Comparable extracts were similarly prepared either omitting the polyamide or replacing it with Polyclar AT (250 mg).

Extraction of Reduced Pyridine Nucleotides

Five g of frozen apple powder, prepared as described above, was mixed with 500 mg of polyamide and suspended in 20 ml of ice-cold 0·1 N KOH in 50 per cent ethanol which had been flushed with nitrogen just prior to use. This suspension was heated for 4 min at 60° with continuous stirring. The extract was centrifuged and the residue re-extracted serially with two 15 ml aliquots of 0·1 N KOH in 50 per cent ethanol at 60° for 4 min. The combined extracts were mixed with a further 100 mg of polyamide, 0·28 ml of 0·75 M tris at pH 9·0 was added and the pH adjusted to 7·8 with ice-cold 2 N HCl. The precipitate which formed was centrifuged off, washed with ice-cold distilled water and the combined supernatant liquids and washings were made to 75 ml. Comparable extracts were prepared either without polyamide or with polyamide replaced by Polyclar AT (500 mg).

The Assay of NAD *and* $NADH_2$

The following reaction mixture was used in the assay finally adopted for both NAD and $NADH_2$: Recrystallized tris buffer, pH 7·4, 54 μmole; ethanol 850 μmole (1700 μmole for $NADH_2$); freshly prepared *N*-methyl phenazonium methosulphate (PMS), 1·5 μmole; resazurin 0·1 μmole in 0·1 ml redistilled methyl cellosolve, extract 0·025 to 0·2 ml containing 20 to 220 $\mu\mu$mole pyridine nucleotide, and water to 3 ml. This reaction mixture was prepared in a 1-cm cuvette and changes in the fluorescence of the mixture were measured in a modified Eppendorf fluorimeter. The filters in the fluorimeter were arranged to give an excitation wavelength of 546 nm, and for fluorescence measurements to be made at wavelengths above 560 nm (filter range 560 to 3000 nm). This fluorescence was measured at an angle of 30° from the primary beam (giving minimal 'scatter' of the primary beam) using an RCA IP21 photomultiplier. The output of the photomultiplier was filtered to reduce 'noise' and attenuated (2500:1) and measured on a Varian G14A-1 recorder. A device was introduced to 'back off' the output of the photomultiplier so that the 1-mV span of the recorder could be used to give high sensitivity. The cuvette, with its contents as described above, was introduced into the cell holder (maintained at a constant 25°) of the fluorimeter and the changes in fluorescence followed for 5 min. During this period fluorescence increased as some of the resazurin was non-enzymically reduced to the fluorescent resorufin; this reduction was always complete within 5 min. At this point 0·02 ml alcohol dehydrogenase (600 μg) was added and the rate of increase in fluorescence followed. After a short lag period, the rate became linear and remained so for 10 min. The rate of dye reduction was measured as the slope of the curve between the third and seventh minute after the addition of the enzyme. Each extract was assayed at two different concentrations and a standard curve using authentic pyridine nucleotides was prepared *with each batch of extracts*. Inhibition tests were also performed with each extract; in these, the rate of reduction of dye due to the presence of particular levels of extract and standard, assayed after being added together, was compared with the rates of dye reduction by the extract and standard, at the same concentrations, assayed separately. The rate of reduction in the combined assay was expressed as a percentage of the predicted rate based on the sum of the rates for the extract and standard assayed separately. Recovery tests were carried out in which various amounts of authentic standards were added to the 5 g lots of frozen tissue prior to extraction.

The Assay of NADP *and* $NADPH_2$

The following reaction mixture was prepared in 1-cm cuvette: recrystallized tris buffer, pH 7·4, 76 μmole; glucose-6-phosphate, 1·65 μmole; PMS freshly prepared 1·5 μmole; resazurin 0·1 μmole in 0·1 ml redistilled methyl cellosolve; EDTA, 1·5 μmole: ethanol, 1700 μmole (only present in the assay for $NADPH_2$): extract, 0·025 to 0·25 ml, containing 20 to 120 $\mu\mu$mole of pyridine nucleotide, and water to a final volume of 3 ml. The non-enzymic reduction of the dye was followed to completion (5 min) at 25° in the Eppendorf fluorimeter. Glucose-6-phosphate dehydrogenase (50 μg in 0·05 ml) was added and the increase in fluorescence followed with time. There was again a short lag phase before the rate of reduction of the dye became linear. As before, the amount of pyridine nucleotide was calculated from the slope of the dye reduction line taken between the third and seventh minute after the addition of the enzyme. As with NAD and $NADH_2$, inhibition tests and standard curves were performed for each batch of extracts. Recoveries of authentic samples of the nucleotides added to the tissue before extraction were also determined.

The standard solutions used for calibration of the assay system were prepared by dilution of concentrated solutions of the nucleotides prepared daily (see Lowry *et al.*[19]). Five mg of nucleotide per 10 ml of 0·02 N HCl for the oxidized forms and 0·02 N KOH for the reduced forms were used. These solutions were diluted immediately before use with distilled water to give a final concentration of 1 μg per ml.

Chemicals

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and alcohol dehydrogenase (yeast) were obtained from Boehringer Corporation (London) Ltd., (catalogue numbers G-6-P-NA 15323, G-6-PDH 15303 and ADH 15418 respectively). PMS and tris were obtained from Koch-Light Ltd., and resazurin from Eastman Kodak Chemical Co. Ltd., Polyclar AT came from Fine Dyestuffs and Chemicals Ltd., Manchester, while the polyamide was a grade produced by M. Woelm of Eschwege, W. Germany for thin layer chromatography.

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