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Studies on the Reduction of Tetrazolium Salts*

II. The Measurement of the Half Reduced and Fully Reduced Formazans of Neotetrazolium Chloride in Tissue Sections

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Summary. 1. The red and purple formazans of NT, deposited in sections as a result of enzyme activity, have absorption characteristics in solution identical to the red intermediate half-formazan and the purple diformazan produced chemically by ascorbate reduction. The red formazan precipitated in sections can be reduced further to the purple diformazan.

2. The total amount of hydrogen generated in a dehydrogenase reaction can be determined by eluting both formazan derivatives into DMF and measuring at a single (isobestic) wavelength.

3. In a solution which contains both formazans, each formazan may be quantified separately from measurements made at two wavelengths, 460 nm and 600 nm. Alternatively, the two formazans can be eluted separately into different solvents.

4. In serial sections reacted for glucose-6-phosphate dehydrogenase, the total activities, determined by each of these elution methods (i.e. measurement at the isobestic wavelength; measurement at two wavelengths; measurement of each component separately), were identical.

5. The total amount of hydrogen, reflected by the total amount of the formazan derivatives, can also be measured directly in the section by scanning and integrating microdensitometry at 585 nm, the isobestic wavelength of the deposited formazans. In addition, the formazans can be separately quantified by measuring at two wavelengths, 520 nm and 620 nm.

6. Identical results were obtained for the dehydrogenase activity of sections determined first by microdensitometry and then by elution and spectrophotometry.

7. An incubation/time curve for glucose-6-phosphate dehydrogenase activity showed that initially the red half-formazan was the main product. The production of this half-formazan began to tail off after about 5 minutes, at which time formation of the purple diformazan increased. The total activity, calculated from the sum of the two formazan derivatives, was linear with time up to at least 12.5 minutes.

Introduction

Tetrazolium salts have long been used to demonstrate dehydrogenase activities in tissue sections; on reduction they are converted to coloured, insoluble formazans. When NT and other ditetrazolium salts are used as the final hydrogen acceptor two products are usually formed in the section; a red component and a more granular bluish or purple component (Farber *et al.*, 1956; Eadie *et al.*, 1970; Altman, 1973; Butcher, 1972). Until recently, however, although it was known that both of these formazans were formed by ascorbate reduction of

* *Abbreviations.* NT 2,2′-(p-diphenylene)-bis (3,5-diphenyl) ditetrazolium chloride. (Neotetrazolium chloride); DMF Dimethyl formamide; MIE Mean integrated extinction; TLC Thin layer chromatography.

pure NT (Okui *et al.*, 1963) only a purple diformazan had been isolated and characterised. Thus, in order to quantify dehydrogenase activities in tissue sections, the formazans were eluted into a suitable solvent (Defendi and Pearson, 1955; Altman, 1969), the absorption measured in a spectrophotometer and the concentration calculated from the extinction coefficient of the purple diformazan alone.

A method has been described for the measurement of the red and purple components in the section by scanning and integrating microdensitometry (Butcher, 1972). The extinction coefficients of these two components in the section were calculated from the concentration of the formazan in the same sections which had been eluted and measured spectrophotometrically. This in turn was determined from the standard curve of the chemically prepared purple diformazan.

In the light of the findings of Altman and Butcher (1973) of a red intermediate half-formazan of NT, with absorption characteristics different from those of the purple diformazan, a revision of the methods of quantitation, both spectrophotometric and microdensitometric, has become necessary, and these are described in the present paper.

Materials and Methods

Red half-formazan and purple diformazan were prepared from NT by the method of Altman and Butcher (1973).

Female albino Wistar rats were killed by asphyxiation under nitrogen. Pieces of liver, up to 4 mm^3 were removed and chilled in hexane at $-70°C$ (Chayen *et al.*, 1973). Sections, 10 μ thick, were cut in a Bright's cryostat, under controlled conditions (Silcox *et al.*, 1965; Chayen *et al.*, 1973).

Fresh unfixed sections were incubated for glucose-6-phosphate dehydrogenase activity at 37°C in an atmosphere of nitrogen in a medium containing 0.05 M glycylglycine buffer pH 8.0; 22% (w/v) polyvinyl alcohol, grade B05/140, (from the Cytochemical Co., 42 Wychwood Avenue, Edgware, Middx, England); 0.3% purified NT (Altman and Butcher, 1973); 5 mM (1.5 mg/ml) G-6-P di Na salt and 3.8 mM (2.5 mg/ml) NADP. To test for NADPH diaphorase activity the G-6-P and NADP were replaced by 3.8 mM (2.5 mg/ml) NADPH. G-6-P, NADP and NADPH were all supplied by Boehringer, Mannheim, Germany.

A Vickers M85 scanning and integrating microdensitometer was used to measure the formazans deposited in the section; a $\times 100$ objective and a scanning spot of 0.25 μ (spot 1) were employed. The density readings of 20 fields of diameter 30 μ, selected at random, were measured in each section and the density readings converted to the mean integrated extinction (see Butcher, 1972).

Formazan in solution was measured in a Hilger and Watts spectrophotometer in microcells of 1 cm path length.

Results

A. Measurement of NT Formazans by Elution and Spectrophotometry

1. Comparison of NT Red Half-Formazan and NT Purple Diformazan Produced by Ascorbate Reduction and by Enzymic Reduction

Some properties of the chemically reduced red half-formazan and that eluted from tissue sections into absolute ethanol are compared in Table 1. Their absorption curves in DMF are shown in Fig. 1. The absorption curve of NT purple diformazan, prepared from NT by ascorbate reduction, is compared in Fig. 2 with that of purple diformazan eluted from sections into DMF.

Table 1. Some properties of chemically and enzymically produced red formazan

	NT red formazan produced by ascorbate reduction	NT red formazan eluted from tissue sections
Absorption maxima in absolute ethanol	249 nm, 312 nm, 500 nm	312 nm, 500 nm[a]
R_f value on TLC	0.81	0.80
Effect of further reduction	purple solution obtained, absorption maximum at 550 nm	purple solution obtained, absorption maximum at 550 nm

[a] Absorption of eluted red formazan increased abruptly below 270 nm due to other materials eluted into ethanol from the tissue section.

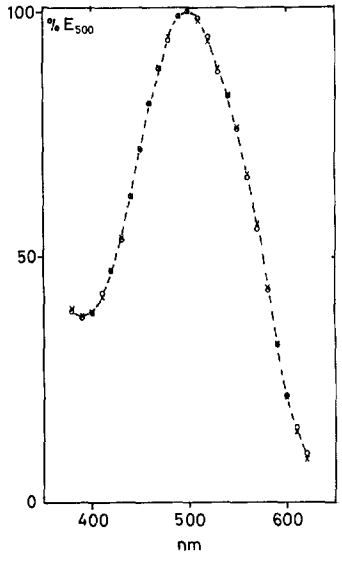

Fig. 1

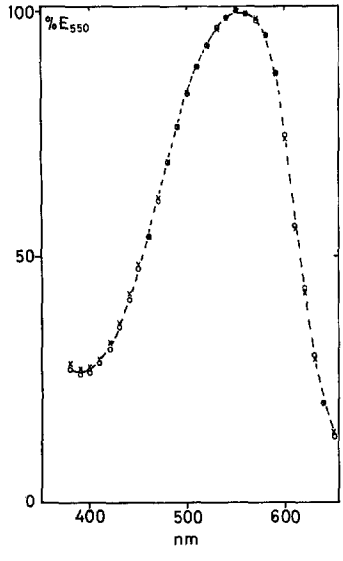

Fig. 2

Fig. 1. The absorption curves of the red formazan in DMF; ×------× chemically prepared by ascorbate reduction of NT; o------o eluted from tissue section

Fig. 2. The absorption curves of the purple diformazan in DMF; ×------× chemically prepared by ascorbate reduction of NT; o------o eluted from tissue section

2. The Molar Extinction Coefficients with Respect to Hydrogen

Altman and Butcher (1973), concluded that NT red formazan is a half reduction intermediate of NT and thus 1 molecule of red formazan requires 1 molecule of hydrogen for its formation from 1 molecule of NT. Its molar extinction coefficient with respect to the hydrogen required for its formation is, therefore, the same as the coefficient of the formazan itself, namely 22000 (Table 2). The purple diformazan, however, requires two molecules of hydrogen for its formation from 1 molecule of NT. The molar extinction coefficient of the purple diformazan is 41000 (Altman and Butcher, 1973); the coefficient with respect to hydrogen is, therefore, half of this value, namely 20500 (Table 2).

Table 2. Molar extinction coefficients of NT formazans in solution with respect to content of hydrogen

Formazan	Solvent	wavelength (nm)	k
Red half-formazan	Ethanol	500	22000
	DMF	460	17900
	DMF	500	22000
	DMF	530	19700
	DMF	600	4700
Purple diformazan	DMF	460	11100
	DMF	530	19700
	DMF	550	20500
	DMF	600	15200

3. Methods of Measurement

a) Measurement at the Isobestic Wavelength. The absorption curves of red half-formazan and purple diformazan in DMF (each at a concentration of 10 nmoles/ml with respect to hydrogen) are shown in Fig. 3. At the isobestic wavelength, 530 nm, each solution gave an optical density of 0.197 (Fig. 3); at this wavelength the molar extinction coefficient, k, (with respect to hydrogen) was, therefore, 19700 (Table 2), irrespective of the nature of the formazan.

b) Measurement at 460 nm and 600 nm. 1 nmole NT red half-formazan/ml DMF gives an extinction of 0.0179 at 460 nm and an extinction of 0.0047 at 600 nm (Fig. 3). The corresponding values for the purple diformazan are 0.0111 at 460 nm and 0.0152 at 600 nm (Fig. 3), all with respect to molar content of hydrogen.

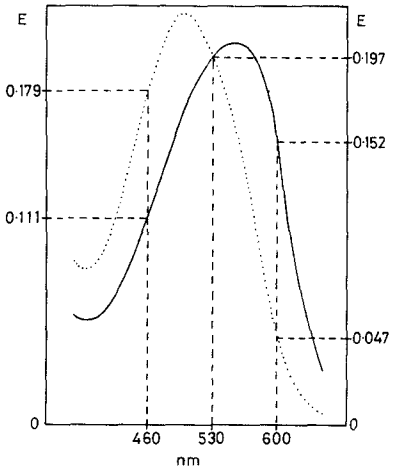

Fig. 3. The absorption curves of the red half-formazan (.....) and the purple diformazan (——) in DMF. The concentration was 10 nmoles/ml in each case, with respect to content of hydrogen

Thus

$$E_{460} \text{ (measured)} = 0.0179x + 0.0111y, \quad (1)$$

and

$$E_{600} \text{ (measured)} = 0.0047x + 0.0152y. \quad (2)$$

Where x = n moles hydrogen (as red half-formazan)/ml DMF and y = n moles hydrogen (as purple diformazan)/ml DMF.

For example, let us suppose that a solution of the formazans eluted from a tissue section has an extinction at 460 nm of 0.400/cm²/ml DMF and an extinction at 600 nm of 0.300/cm²/ml DMF.

Then

$$0.400 = 0.0179x + 0.0111y, \quad (3)$$

and

$$0.300 = 0.0047x + 0.0152y. \quad (4)$$

Multiplying Eq. (3) by 1.37, this becomes

$$0.548 = 0.0246x + 0.0152y \quad (5)$$

$$\therefore 0.548 - 0.300 = 0.0246x - 0.0047x$$

$$0.248 = 0.0199x$$

$$x = 12.5.$$

Substituting in Eq. (3). Then

$$0.400 = 0.223 + 0.111y$$

$$y = 15.9.$$

Thus this section contains 12.5 n moles hydrogen (as red half-formazan)/cm² and 15.9 n moles hydrogen (as purple diformazan)/cm².

The total hydrogen trapped can then be calculated by the addition of the two components. In the above example this would be 12.5 + 15.9 = 28.4 n moles hydrogen/cm².

c) By Two Separate Elutions. Since the red intermediate is soluble in ethanol (Altman and Butcher, 1973) it can be extracted from the section into this solvent leaving the purple diformazan in the section. 10 n moles red formazan (in terms of hydrogen)/ml ethanol gives an extinction value of 0.220 at 500 nm (Altman and Butcher, 1973). The purple diformazan can then be eluted into DMF. At 550 nm, an extinction of 0.205 is equivalent to 10 n moles purple diformazan (in terms of hydrogen)/ml DMF (Fig. 3). The total hydrogen trapped can then be calculated by addition.

4. Comparison of Methods of Measurement

12 serial sections of rat liver were incubated for glucose-6-phosphate dehydrogenase activity; 6 sections were incubated for 5 min, 6 for 10 min. After incubation the area of each section was measured by planimetry of the image of the section projected through a photographic enlarger. The formazans from each of 3 sections incubated for 5 min and for 10 min were eluted into DMF. The extinction values of each solution were measured at 460 nm, 530 nm and 600 nm (Table 3). The red half-formazan was eluted from each of the other 6 sections

Table 3. The measurement of the extinctions of formazans eluted from tissue sections

Section No.	Incubation time (min)	E/ml solvent/cm²				
		A. DMF			B. Ethanol and DMF	
		E_{460}	E_{530}	E_{600}	E_{500} (ethanol)	E_{550} (DMF)
1	5	0.312	0.436	0.232	—	—
2	5	0.316	0.446	0.244	—	—
3	5	0.324	0.461	0.254	—	—
4	5	—	—	—	0.215	0.288
5	5	—	—	—	0.210	0.276
6	5	—	—	—	0.213	0.256
7	10	0.635	0.950	0.575	—	—
8	10	0.595	0.895	0.544	—	—
9	10	0.611	0.930	0.541	—	—
10	10	—	—	—	0.326	0.679
11	10	—	—	—	0.316	0.656
12	10	—	—	—	0.291	0.690

Table 4. The determination of the amount of hydrogen trapped in a section (from the data given in Table 3)

Incubation time (min)	n moles hydrogen/cm²						
	as red half-formazan		as purple diformazan		total hydrogen		
	from E_{460} and E_{600}	from E_{500}	from E_{460} and E_{600}	from E_{550}	from E_{460} and E_{600}	from E_{530}	from E_{500} and E_{550}
5	9.6 ± 0.2	9.7 ± 0.1	13.0 ± 0.7	13.5 ± 0.8	22.7 ± 0.7	22.9 ± 0.7	23.2 ± 0.8
10	14.4 ± 0.6	14.1 ± 0.8	32.1 ± 1.2	32.9 ± 0.8	46.5 ± 1.5	47.1 ± 1.4	47.0 ± 0.8

into absolute ethanol and the extinction values of the solutions at 500 nm were recorded. These sections were then dried before elution of the purple diformazan into DMF and measurement of the extinction values at 550 nm (Table 3). The concentration of hydrogen trapped in each section was calculated from the above data (Fig. 3, Table 3). These results are shown in Table 4.

5. The Formation of Red Half-Formazan and Purple Diformazan as a Function of Incubation Time

10 μ sections of rat liver were incubated for various times for glucose-6-phosphate dehydrogenase activity. The separate amounts of red half-formazan and purple diformazan were determined, in terms of hydrogen, by elution into ethanol and DMF, as described in Section 3 (c). The results are shown in Fig. 4. Initially the red half-formazan was produced. However, as its rate of formation decreased, the rate of production of the purple diformazan increased (Fig. 4). The total activity, obtained by addition of the two components, was linear with incubation time.

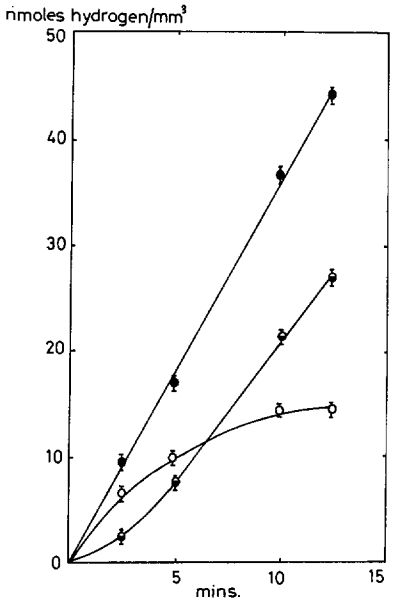

Fig. 4. Incubation/time curves for glucose-6-phosphate dehydrogenase in rat liver sections. o——o red half-formazan, in terms of hydrogen; ⊖——⊖ purple diformazan, in terms of hydrogen; •——• total hydrogen

B. Measurement of NT Formazans in the Section by Scanning and Integrating Microdensitometry

1. Determination of Molar Extinction Coefficients of NT Red Formazan and NT Purple Diformazan (in Terms of Hydrogen)

a) Theoretical Considerations. The extinction coefficient of a compound in solution is determined from the Beer-Lambert equation; $E = kcl$.

Where E = extinction
k = molar extinction coefficient
c = concentration in moles/1000 cc
l = path length in cm.

In scanning and integrating microdensitometry, the path length, l, is the thickness of the section. Unless this is known accurately, the above equation cannot be used to determine the extinction coefficient of a compound deposited in a section.

However,

$$c = \frac{M}{V}$$

Where M = mass in moles and V = volume in 1000 cc.

Furthermore

$$V = Al.$$

Where A = Area in 1000 cm^2, and l = length in cm.

Table 5. Determination of the molar extinction coefficient (k) of NT red half-formazan in the section

M.I.E.$_{510\ nm}$ "E"	Area (1000 cm^2) "A"	Moles hydrogen "M"	k	$\left(=\frac{EA}{M}\right)$
0.113	3.20×10^{-4}	1.60×10^{-9}	22600	
0.150	3.16×10^{-4}	1.97×10^{-9}	24100	
0.232	3.24×10^{-4}	3.28×10^{-9}	22800	22800 ± 780
0.253	3.24×10^{-4}	3.64×10^{-9}	22500	
0.334	3.17×10^{-4}	4.80×10^{-9}	22000	

Substituting in the Beer-Lambert equation, then

$$E = \frac{kMl}{Al} = \frac{kM}{A}$$
$$\therefore k = \frac{EA}{M} \quad (6)$$

Thus the extinction coefficient can be determined without knowledge of the thickness of the section.

b) Red Formazan. The absorption maximum of NT red half-formazan measured in the section by microdensitometry is 510 nm (Butcher, 1972). The M.I.E. at this wavelength was recorded in sections incubated for different times and containing only the red formazan (by removing the purple diformazan in benzene). The areas of the sections were measured by planimetry, the formazans were eluted into DMF and the mass of formazan, with respect to hydrogen, determined in each section (Table 5). The molar extinction coefficient of the red half-formazan in the section was then calculated by substituting in Eq. (6) (Table 5).

c) Purple Diformazan. Sections incubated for different times, and containing only the purple diformazan (by removal of the red half-formazan in ethanol) were prepared. The molar extinction coefficient at 550 nm, the absorption maximum of the purple diformazan (Butcher, 1972), was then determined in the same way as described above. The results are shown in Table 6.

Table 6. The determination of the molar extinction coefficient (k) of the purple diformazan in the section

M.I.E.$_{550nm}$ "E"	Area (1000 cm^2) "A"	Moles hydrogen "M"	k	$\left(=\frac{EA}{M}\right)$
0.106	3.21×10^{-4}	4.49×10^{-9}	7580	
0.130	3.32×10^{-4}	5.68×10^{-9}	7600	
0.180	3.16×10^{-4}	7.65×10^{-9}	7460	7570 ± 90
0.213	3.22×10^{-4}	8.89×10^{-9}	7710	
0.310	3.22×10^{-4}	13.2×10^{-9}	7560	
0.431	3.18×10^{-4}	18.3×10^{-9}	7500	

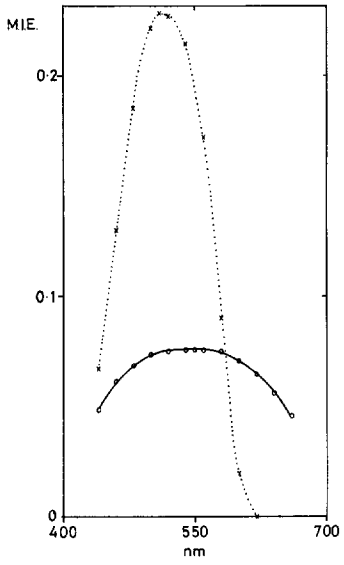

Fig. 5. The absorption curves, measured by microdensitometry in the section of the red and purple formazans of NT; ×·····× red half-formazan; o·····o purple diformazan. The concentration was 10 nmoles/ml in each case, with respect to the content of hydrogen

2. Absorption Curves of the Formazans in the Section

The absorption curves of the red half-formazan and of the purple diformazan were determined by scanning and integrating microdensitometry. Knowing the molar extinction coefficient, in the section, of each formazan (Tables 5 and 6), the absorption curves of the formazans, each at a concentration of 10 n moles hydrogen/cm^2, were calculated (Fig. 5). At the isobestic wavelength, 585 nm, the molar extinction coefficient was 7,400 (Fig. 5); at this wavelength the M.I.E. should be directly proportional to the amount of hydrogen trapped, irrespective of the nature of the formazan present in the section.

3. The Measurement at 585 nm of Hydrogen Trapped in the Section

Serial sections of rat liver were incubated for NADPH diaphorase activity for 2, 4, 6, 8 and 10 min. The M.I.E. at 585 nm was recorded for each section and the amount of hydrogen trapped/cm^2 of tissue calculated (Table 7). The formazans in these sections were eluted into DMF and their extinction values at 530 nm were determined in a spectrophotometer. The activities calculated from these values are compared with those obtained by scanning and integrating microdensitometry in Table 7.

4. The Separate Measurement of Red Half-Formazan and Purple Diformazan in Sections

The red half-formazan and the purple diformazan can be separately quantified in sections by two measurements, one at 520 nm and a second at 620 nm. The procedure has been described by Butcher (1972).

Table 7. A comparison of the determination of enzyme activity by microdensitometry and by elution and spectrophotometry

Incubation time (min)	M.I.E.$_{585\,nm}$	E_{530}/cm^2/ml DMF	n moles hydrogen/cm^2	
			Microdensitometry ($k_{585} = 7400$)	Spectrophotometry ($k_{530} = 19700$)
2	0.102	0.262	13.8	13.3
4	0.206	0.545	27.8	27.6
6	0.322	0.846	43.5	43.0
8	0.405	1.092	54.7	55.4
10	0.511	1.370	69.0	69.5

5. The Calculation of the Amount of Hydrogen in a Specific Area of a Section

In order to determine the molar extinction coefficients of the formazans deposited in the section, it was necessary to use the Beer-Lambert equation in a revised form, namely $k = \frac{EA}{M}$ (see Section B I(a)). Therefore

$$M = \frac{EA}{K} \tag{7}$$

The enzyme activity of any individual part of a tissue section can be calculated from Eq. (7) if the M.I.E. at 585 nm and the area are known. For example, let us suppose that a specimen of diameter 20 μ has a M.I.E.$_{585}$ of 0.1.

Then $E = 0.1$

$A = 314\ \mu^2 = 3.14 \times 10^{-11}\ 1000\ cm^2$

k = molar extinction coefficient at 585 nm = 7400.

Substituting in Eq. (7), then

$$M = \frac{0.1 \times 3.14 \times 10^{-11}}{7400} \text{ moles hydrogen}$$

$$= 4.24 \times 10^{-14} \text{ moles hydrogen}$$

$$= 42.4 \text{ f moles hydrogen.}$$

Thus a specimen of 20 μ diameter, giving an extinction at 585 nm of 0.1, contains 42.4 f moles hydrogen. It is not possible to calculate the concentration of hydrogen unless the thickness of the section is known. However, this can be determined chemically in the same section after removal of the formazans (Butcher, 1971).

Discussion

Previously, methods had been described for the determination of the concentration of NT formazan in a tissue section, both in situ by scanning and integrating microdensitometry (Butcher, 1972) and by elution and spectrophotometry (Defendi and Pearson, 1955; Altman, 1969). Whilst it has been known for some time that two forms of formazan are deposited in the section, only the purple diformazan had been positively identified chemically. The calculation of enzyme

activity relied, therefore, solely on the absorption properties of this purple diformazan in solution. The isolation and characterisation of a red half-formazan intermediate of NT by Altman and Butcher, (1973) necessitated a review of the methods of calculation of enzyme activity in tissue sections,

The data in Table 1 and Fig. 1 show that the red half-formazan has the same spectral and chromatographic properties, whether it is prepared by chemical reduction or by enzymatic reduction in sections. Similarly the absorption characteristics of the purple diformazan prepared from the chemical reduction of NT and the purple formazan eluted from sections are identical (Fig. 2). It was not possible to obtain a sufficient quantity of the two formazans from tissue sections for any analytical work, but it seems reasonable to assume that these compounds are identical to the two compounds produced chemically from NT by ascorbate reduction and analysed in detail by Altman and Butcher (1973).

It is usual to express enzyme activities obtained from elution and spectrophotometry in terms of weight of formazan deposited in the section. However, this can be misleading because the same amount of dehydrogenase activity can produce various amounts of different formazans, depending on the molecular weight of the formazan formed. Moreover, monoformazans require only 1 molecule of hydrogen for their formation from 1 molecule of tetrazolium salt whereas diformazans require two molecules. Results are, therefore, better expressed in terms of the moles of hydrogen per unit mass or volume of tissue used in the formation of the formazan (Altman, 1969). This has the additional advantage in that enzyme activities determined in tissue sections can then be compared with those obtained from more conventional biochemical studies (Altman, 1972).

The calculation of results in this way becomes essential where the two formazans of NT are present in the same section, particularly since the red intermediate half-formazan requires only 1 molecule of hydrogen for its formation from NT whereas the purple diformazan requires 2. The molar extinction coefficients, in solution, of these two formazans with respect to the amount of hydrogen required for their formation have, therefore, been calculated (Table 2, Fig. 3).

From this information, three methods of determining the amount of formazan in a solution have been tested. The simplest method, a single measurement of the eluted formazan at the isobestic wavelength, 530 nm, permits only the total concentration of formazan hydrogen to be determined (Tables 3 and 4). This is sufficient for most quantitative studies. However, by measuring at two wavelengths, 460 nm and 600 nm, the individual concentrations of red intermediate formazan hydrogen and purple diformazan hydrogen in the same solution can be calculated (Tables 3 and 4). This is also possible by differentially removing the red intermediate into absolute ethanol and then eluting the remaining purple diformazan into DMF (Tables 3 and 4). In serial sections reacted for glucose-6-phosphate dehydrogenase activity, the concentration of red formazan, determined by these two methods, were very similar; so too were the concentrations of purple diformazan (Table 4). Moreover the sum of these two individual activities resulted in an activity of the same order as that obtained from a single measurement at the isobestic wavelength (Table 4).

A method for the determination of the amount of formazan within a section by scanning and integrating microdensitometry, and thus without recourse to

elution, has previously been described (Butcher, 1972). In that paper the extinction coefficients of the red and purple formazans were calculated from a single extinction coefficient of formazan in solution of 20000. Since the individual extinction coefficients in solution of the two formazans with respect to hydrogen are very similar (22000 at 500 nm for the red formazan; 20500 at 550 nm for the purple diformazan) the findings of that paper remain substantially correct. The isobestic wavelength remains at 585 nm; the molar extinction coefficient of the formazan hydrogen at that wavelength is 7400 (Fig. 5). The red and purple formazans can be separately measured by taking readings at 520 nm and 620 nm (Butcher, 1972). It is of interest that the extinction coefficient of the red half-formazan is the same in the section and in solution (Tables 2 and 5), whereas the extinction coefficient of the diformazan is very much lower when precipitated in sections (Tables 2 and 6). This is probably because the half-formazan is diffusely distributed in the sections and the diformazan is present as a particulate precipitate.

A comparison of enzyme activities in the same sections determined by measurement at the isobestic wavelength (585 nm for formazan in the section measured by scanning and integrating microdensitometry; 530 nm for formazan in solution, measured by spectrophotometry) is shown in Table 7. The activities were very similar. Moreover, in each case, enzyme activity was directly proportional to the time of incubation (Table 7). In addition, the incubation/time curves (Fig. 4) show that the kinetics of the formation of the red half-formazan and the purple diformazan are very different. However, the total activity, in terms of hydrogen, is linear with respect to incubation time. Such an analysis emphasises the importance of not disregarding the red half-formazan, and the common practice of removing this in acetone prior to examination of the sections should be avoided.

Thus it is possible to determine the activity of a section containing both red and purple formazans, either by scanning and integrating microdensitometry or by elution and spectrophotometry. A single measurement at the isobestic wavelength gives the total activity; two measurements at different wavelengths give the separate amounts of red and purple formazans.

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