STUDIORUM PROGRESSUS

Substantivity and other Factors Responsible for Formazan Patterns in Dehydrogenase Histochemistry

Introduction. The superiority of some newer tetrazolium salts for the visualisation of reductase activity at the cellular level is due mainly to two factors. First, a relatively high reduction potential of the tetrazole and secondly, the property of forming dense, insoluble, non-crystalline formazan pigments[1]. The localisation of formazan in tissue sections, and the possibility of staining enzymically active intracellular organelles, has been assumed to depend on certain properties of the final pigment, namely insolubility and binding to proteins. The latter quality has been described as substantivity for protein. With the exception of a report by WATTENBERG[2], no further reference has been made to binding capacities of tetrazolium compounds to tissue constituents and the possibility that pigment distribution may depend on adsorption of the dye-precursor (indicator) seems largely to have been neglected.

With this factor in mind we examined the substantive properties of a number of mono- and di-tetrazoles and were able to demonstrate a strongly enhanced adsorption, particularly to lipoprotein structures, of compounds bearing polar groupings at the 2-position. It appeared possible that this property could determine the intracellular distribution of enzymically produced formazan pigment.

Materials and Methods. Fresh frozen sections, 2 μ in thickness, cut on a cryostat and 3 μ frozen sections from tissue fixed for 16 h at 4° in formol-calcium were incubated for various periods of time at 20° in solutions of tetrazolium salt in buffer containing 0.1 mg salt per ml. After incubation, the sections were washed in three changes of buffer and distilled water for 30 min and immersed for 2 min in 0.12 *M* sodium carbonate solution containing 0.1 *M* ascorbic acid (pH 8.5). The influence of pH on tetrazole adsorption and elution was tested using 0.1 *M* acetate buffer pH 2–5.5; 0.06 *M* phosphate buffer pH 5–8; 0.2 *M* borate buffer pH 7.5–9. The following tetrazolium salts were tested: (Tab. I and II).

In order to achieve enzymic reduction of tetrazole, Nitro-BT (XIII) and MTT (I) were incorporated in media and used for the demonstration of reduced diphosphoyridine nucleotide (DPNH)-tetrazolium reductase and lactate dehydrogenase in formalin fixed[3] or cryostat sections[4,5]. The latter were also stained for succinate dehydrogenase and β-hydroxybutyrate dehydrogenase activity using MTT-Co[6] or Nitro-BT[4]. Bound tetrazole after pre-treatment of sections with Nitro-BT was reduced enzymically according to WATTENBERG[2].

Experiments similar to those performed on tissue sections were carried out on samples of rat liver microsomes prepared according to POPJÁK et al.[7], and on rat liver mitochondria prepared in 0.35 *M* sucrose[8]. Both microsomes and mitochondria were used in suspension and as smear preparations.

Samples of purified lipid fractions and proteins, adsorbed to filter paper (Whatman No. 1), to cellulose acetate membranes, or affixed on glass slides, were treated with tetrazolium solution and after washing subjected to alkaline reduction. The following substances were tested: glyceryl triolate, diolein, triolein, glyceryl tristearate, phosphoryl choline chloride, lecithin, cephalin, ganglioside, cerebroside, sphingomyelin, cholesterol stearate, 2-methyl-3-phytyl-1:4-naphthoquinone (vitamin K_1), 2-methyl-1:4-naphthoquinone (vitamin K_3), 2-methyl-3-hydroxy-1:4-naphthoquinone (phthicocol), serum albumin, γ-globulin (bovine), ovalbumin.

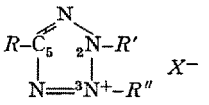
Results. Mild alkaline reduction produced formazan staining of tissue sections, isolated mitochondria and microsomes which had been pre-treated with any one of the ditetrazoles listed above. The most intense staining was obtained with Nitro-BT and its non-crystalline formazan pigment revealed intracellular structures distinctly. Similar staining was achieved by alkaline reduction of tissue sections pre-treated with the monotetrazolium salts INT (III), M and B 1762 A (VII) and 1767 (IV). The other monotetrazoles tested (I, II, V, VI, VIII, IX, X) were not bound by tissue structures and no staining was obtained when the washed sections were placed in the reducing solution. The adsorption of Nitro-BT was a rapid process. At pH 7, this tetrazolium salt was retained by lipoprotein structures after 5 sec treatment, and after 90 sec treatment dense staining of intracellular structures was obtained upon subsequent reduction. Incubation for 3 min resulted in maximum adsorption of tetrazole. Substantive mono- and di-tetrazoles were bound to tissue components at neutral and alkaline pH; below pH 5, adsorption did not occur. Adsorbed tetrazole was eluted by acidic buffer solutions. In contrast to tissue sections, purified lipids adsorbed tetrazole within the acid range.

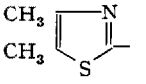
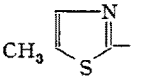
Formalin fixation of tissue increased the binding of substantive tetrazoles, especially Nitro-BT, and lipoprotein structures became distinctly outlined on development of the formazan pigment. In rat kidney tubular cells, for example, the mitochondrial pattern was revealed clearly (Fig. 1). Infoldings of the basal cell membranes[9] were heavily stained and could be distinguished from the mitochondria by their looplike arrangement (Fig. 2). Nuclear membranes were outlined and red cell envelopes irregularly stained after tetrazole treatment for longer than 90 sec. The staining pattern obtained by enzymic reduction of Nitro-BT using, for example, DPNH as a substrate was very similar to that obtained on non-enzymic reduction of adsorbed Nitro-BT (Fig. 3).

Non-substantive and some substantive tetrazolium salts, after reduction by tissue enzyme systems, give rise to particulate formazans. In most cases these are easily recognisable as crystals. An exception is MTT-Co where the particulate end product is a round deposit of metal formazan of high density, which bears no resemblance to the easily produced crystals of this complex. The particulate pigment is distributed at regular intervals along active mitochondria, yielding a dot-like or (under favourable conditions) a spiral-like pattern[10]. This pattern is preserved in formalin-fixed tissue (Fig. 4). Enzymic

[1] A. G. E. PEARSE, *Histochemistry, Theoretical and Applied*, 2nd Ed. (Churchill, London 1960).
[2] L. W. WATTENBERG, Proc. Soc. exp. Biol. Med. *87*, 535 (1954).
[3] A. B. NOVIKOFF and B. MASEK, J. Histochem. Cytochem. *6*, 217. (1958).
[4] M. M. NACHLAS, D. G. WALKER, and A. M. SELIGMAN, J. biophys. biochem. Cytol. *4*, 29 (1958).
[5] D. G. SCARPELLI, R. HESS, and A. G. E. PEARSE, J. biophys. biochem. Cytol. *4*, 747 (1958).
[6] R. HESS, D. G. SCARPELLI, and A. G. E. PEARSE, J. biophys. biochem. Cytol. *4*, 753 (1958).
[7] G. POPJÁK, L. GOSSELIN, I. Y. GORE, and R. G. GOULD, Biochem. J. *69*, 238 (1958).
[8] W. C. SCHNEIDER and G. H. HOGEBOOM, J. biol. Chem. *183*, 123 (1950).
[9] J. RHODIN, Intern. Rev. Cytol. *7*, 485 (1958).
[10] A. G. E. PEARSE and D. G. SCARPELLI, Nature *181*, 702 (1958).— D. G. SCARPELLI and A. G. E. PEARSE, Anat. Rec. *132*, 133 (1958).

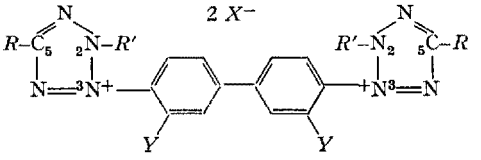
Tab. I. Monotetrazolium salts



	Compound	R	R'	R''	X^-	Substantivity[a]
I	MTT[b]	C_6H_5	C_6H_5		Br	0
II	5-MTT[b]	C_6H_5	C_6H_5		Br	0
III	INT[c]	C_6H_5	p-I-C_6H_4	p-NO_2-C_6H_4	Cl	++
IV	M and B 1767[d]	C_6H_5	C_6H_5	p-$C_6H_5CH=CHC_6H_4$	Cl	+
V	M and B 1689A[e]	C_6H_5	C_6H_5	p-$C_6H_5N=NC_6H_4$	I	0
VI	M and B 1795[e]	p-HOC_6H_4	C_6H_5	p-$C_6H_5N=NC_6H_4$	Cl	0
VII	M and B 1762A[e]	C_6H_5	C_6H_5	α-p-$C_6H_5N=NC_{10}H_6$	I	++
VIII	BCT[f]	C_6H_5	C_7H_4NS	$HOOCC_6H_4$	–	0
IX[g]		C_6H_5	C_7H_4NS	p-NO_2-C_6H_4	Br	0
X	TTC[h]	C_6H_5	C_6H_5	C_6H_5	Cl	0

[a] Substantivity for fresh frozen or formalin fixed sections. Conditions see text.
[b] H. Beyer and T. Pyl, Ber. dtsch. chem. Ges. *87*, 1505 (1954).
[c] S. W. Fox and E. H. Atkinson, J. Amer. chem. Soc. *72*, 3629 (1950).
[d] A. W. Nineham, D. L. Pain, and R. Slack, J. chem. Soc. *1954*, 1568.
[e] A. W. Nineham, D. L. Pain, and R. Slack, J. chem. Soc. *1954*, 1565.
[f] G. G. Glenner, personal communication.
[g] S. S. Karmarkar, A. G. E. Pearse, and A. M. Seligman, J. org. Chem. *25*, 575 (1960).
[h] H. von Pechmann and P. Runge, Ber. dtsch. chem. Ges. *27*, 2920 (1894).

Tab. II. Ditetrazolium salts



	Compound	R	R'	Y	X^-	Substantivity[a]
XI	BT[l]	C_6H_5	C_6H_5	OCH_3	Cl	+
XII	NT[k]	C_6H_5	C_6H_5	–	Cl	+
XIII	Nitro-BT[l]	C_6H_5	p-NO_2-C_6H_4	OCH_3	Cl	++++
XIV	Tetranitro BT[l]	p-NO_2-C_6H_4	p-NO_2-C_6H_4	OCH_3	Cl	++
XV[g]		C_6H_5	C_7H_4NS	OCH_3	Cl	+

[a] Substantivity for fresh frozen or formalin fixed sections, Conditions see text.
[i] A. M. Rutenburg, R. Gofstein, and A. M. Seligman, Cancer Res. *10*, 113 (1950).
[k] W. Antopol, S. Glaubach, and L. Goldman, Publ. Health Rep. Wash. *63*, 1231 (1948).
[l] K. C. Tsou, C. S. Cheng, M. M. Nachlas, and A. M. Seligman, J. Amer. chem. Soc. *78*, 6139 (1956).

formation of metal-formazan particles is not confined to structurally intact mitochondria but occurs also with submitochondrial fragments[11] and, in the case of microsomal enzymes, with agglutinated microsomal particles. Fig. 5 shows the particulate formazan pattern of MTT-Co produced by microsomal DPNH-tetrazolium reductase activity of a smear preparation of rat liver microsomes. A similar pattern was obtained with reduced triphosphopyridine nucleotide as a substrate. Microsomal smears were stained as a structureless layer by Nitro-BT regardless of whether the diformazan was produced enzymically or non-enzymically (Fig. 6). When using MTT methods for the demonstration of strictly mitochondrial enzymes, as succinate[12], β-hydroxybutyrate[13] or α-glycerophosphate dehydrogenase[14], active sites are most numerous in cellular areas where the mitochondria are numerous. In the epithelia of rat epididymis and vas deferens, for example, reactions for these enzymes were restricted to the luminal portion of the cells. This region can be shown to contain most of the mitochondria[15] (Fig. 7–9). Clear distinction

[11] A. G. E. Pearse, D. G. Scarpelli, and R. Hess, J. biophys. biochem. Cytol. *7*, 243 (1960).
[12] G. H. Hogeboom, W. C. Schneider, and G. E. Pallade, J. biol. Chem. *172*, 619 (1948).
[13] A. L. Lehninger, H. C. Sudduth, and J. B. Wise, J. biol. Chem. *235*, 2450 (1960).
[14] R. L. Ringler and T. P. Singer, Arch. Biochem. Biophys. *77*, 229 (1958).
[15] N. Shor, personal communication.

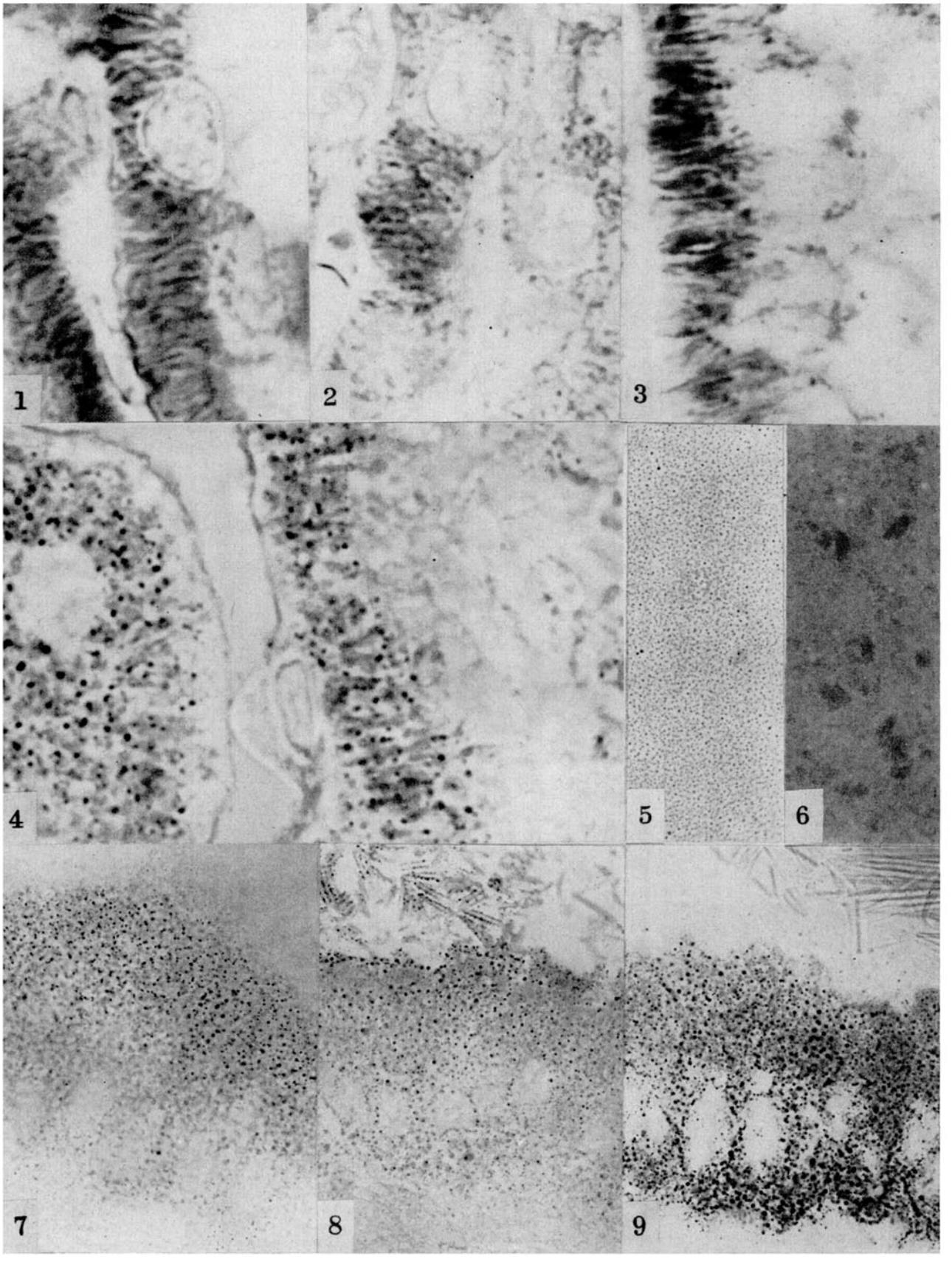

1
2
3
4
5
6
7
8
9

Fig. 1. 3 μ frozen sections of proximal tubule of rat kidney. Staining of both membranous and mitochondrial structures by diformazan produced by alcaline reduction of substantive Nitro-BT. ×2450.
Fig. 2. Same section as Figure 1. Non-enzymic formazan staining of infoldings of cytoplasmic membrane. Distal tubule ×2450.
Fig. 3. Serial section to Figure 1. Enzymic reduction of Nitro-BT by DPNH-diaphorase reaction results in a staining pattern very similar to that obtained on reduction of adsorbed ditetrazole by ascorbate. ×2450.
Fig. 4. DPNH-diaphorase reaction on cells of proximal tubule from formalin-fixed rat kidney using MTT-Cobalt as electron acceptor. Particulate metal-formazan is deposited in mitochondrial areas. ×2450.
Fig. 5. Particulate monoformazan obtained by incubating rat liver microsomes in a medium containing MTT, Co^{++} and DPNH as a substrate. ×1000.
Fig. 6. Diffuse enzymic staining of microsomal aggregates by Nitro-blue formazan. DPNH-diaphorase reaction. ×1000.
Fig. 7. Epithelia of rat vas deferens. α-glycerophosphate dehydrogenase reaction using a MTT-Co method, 5 min incubation. Particulate formazan is deposited in mitochondria-rich portions facing the lumen. 2 μ cryostat section. ×1000.
Fig. 8. Serial section to Figure 7. Demonstration of β-hydroxybutyrate dehydrogenase activity in luminal parts of vas deferens epithelia and in mitochondrial equivalents composing the midpiece of spermatozoa. ×1000.
Fig. 9. DPNH-diaphorase reaction. Metal formazan is distributed throughout the vas deferens cells due to the joint activities of both mitochondrial and microsomal structures. The latter are mainly confined to the basal portions of the cells. ×1000.

between luminal and basal regions was obtained only when MTT was used as electron acceptor. The substantive Nitro-BT produced a uniform staining of the whole cell with formazan and after incubation for more than 15 min detail was lost due to crystallisation along numerous fat vacuoles (Fig. 10, 11). With DPNH as a substrate, particulate MTT-Co formazan was deposited throughout the cells. This appearance is considered to be due to a participation of both mitochondrial and microsomal tetrazolium reductase activity.

In order to exclude the possibility that the distribution pattern of reductase activity observed is peculiar to tetrazole as final electron acceptor, cryostat sections of vas deferens were incubated in media containing dehydrogenase substrate and Janus Green B as indicator. Media for the demonstration of succinate, α-glycerophosphate and DPNH reductase activity were prepared as a modification of the method of COOPERSTEIN, DIXIT and LAZAROW[16]. The reduction of Janus Green B to its leuco derivative in vas deferens epithelia closely followed the pattern described above for MTT-reductase activity. This result does not support the view that, in tissue sections, Janus Green B can be used to distinguish mitochondria from microsomes[16].

Lipoprotein structures devoid of mitochondrial matrix but intimately associated with mitochondria became uniformly stained in formalin-fixed or cryostat sections incubated in media containing Nitro-BT for demonstrating dehydrogenase activity. Examples of such structures are: Protein absorption droplets in the nephron (Fig. 12, 13), motor-end plates on enzymically active muscle fibres and myelinated nerve fibres in voluntary muscle (Fig. 14). Reactions for the mitochondrial enzymes succinate dehydrogenase and β-hydroxybutyrate dehydrogenase were capable of staining such structures, although to a lesser degree than reactions for partially soluble enzymes as, for instance, lactate dehydrogenase. Nuclear membranes required an incubation time longer than 30 min to become enzymically stained. Strong enzymic and non-enzymic staining with Nitro-BT of cytoplasm and dentritic fibres was obtained with the lipid rich central nervous system (Fig. 15).

Due to the effect of freezing and thawing, fragmentation of mitochondria and membrane structure was apparent in thin cryostat sections stained with diformazan of Nitro-BT (Fig. 16). Gross mitochondrial structure was preserved and well demonstrated in parallel sections which were postchromed by the method of ELFTMAN[17] and stained for phospholipids with Luxol fast blue, Sudan black or Solochrome cyanine R (Fig. 17).

Binding of Nitro-BT and other ditetrazoles to both formalin-fixed and frozen-thawed sections, was reduced by chromium mordanting at 60°[17] or by oxidation with $KMnO_4$. Cryostat sections, postfixed in formalin and subsequently treated with Nitro-BT, revealed a globular staining pattern on reduction and loss of mitochondrial detail. Adsorption of Nitro-BT to lipid structures was abolished by the presence of polyvinylpyrrolidone (PVP) 7.5%, dextran 6%, or methylcellulose 7.5% in the medium, presumably by competitive interaction of these substances. Sucrose (0.88 *M*) slowed down binding to lipids, without visibly altering the substantive properties of tetrazoles.

With the exception of glyceryl tristearate, all the lipid fractions tested were capable of binding Nitro-BT, lecithin being most active. Less polar ditetrazoles combined weakly, and non-substantive monotetrazoles were not adsorbed. Phthiocol, above pH 6.5, formed water-soluble red complexes with all the tetrazoles examined. Of the crystalline proteins tested, only γ-globulin bound Nitro-BT to a slight degree. Under the conditions used, Nitro-BT and other ditetrazoles were adsorbed weakly to supporting cellulose, or cellulose acetate.

Discussion. A survey of a limited number of tetrazolium salts has shown that preferential adsorption of tetrazole by lipoprotein-containing cell structures may be related to a) the planar ditetrazole molecule containing the substituted benzidine nucleus and b) to polar groupings attached to the 2-position of the tetrazole ring. The first property puts ditetrazoles (and also monotetrazoles bearing large benzeneazo-substituents in the 3-position) in a group of substantive compounds related to direct dyes for cellulose[18] of the general type: $R_1-N:N-X-N:N-R_2$. Substantivity for lipoprotein, however, seems mainly to be conferred on the molecule by the second mentioned factor. This would explain the substantive properties of the monotetrazole INT. Substantivity is greatly enhanced by a combination of a) and b) as is the case with Nitro-BT. Competing electro-negative groups in position 5 apparently are capable of inhibiting the effect of similar groups in the 2-position; tetra-Nitro-BT is less substantive than Nitro-BT. The binding of the tetrazoles to tissue structures seems to be due to weak electrostatic forces, as adsorption is readily reversed by changes in pH. It is likely that the effective 2-*p*-nitrophenyl radical is present as pseudo-acid since it interacts with tissue at neutral or alkaline pH.

The term 'substantivity' should be used only in a broad sense with reference to histochemical systems. In dye chemistry, substantivity is defined as the property of a compound to become adsorbed by a fibrous substrate from a solution[19], and, quantitatively, is determined as adsorbability minus desorbability[20].

[16] S. J. COOPERSTEIN, P. K. DIXIT, and A. LAZAROW, Anat. Rec. *138*, 49 (1960).
[17] H. ELFTMAN, J. Histochem. Cytochem. *2*, 1 (1954).
[18] T. VICKERSTAFF, *The Physical Chemistry of Dyeing*, 2nd Ed. (Oliver & Boyd, London 1954).
[19] J. WEGMANN, Textil-Rundschau 1959, No. 11.
[20] P. RUGGLI, J. Soc. Dyers & Col. Jubilee Vol. 1934, p. 77.

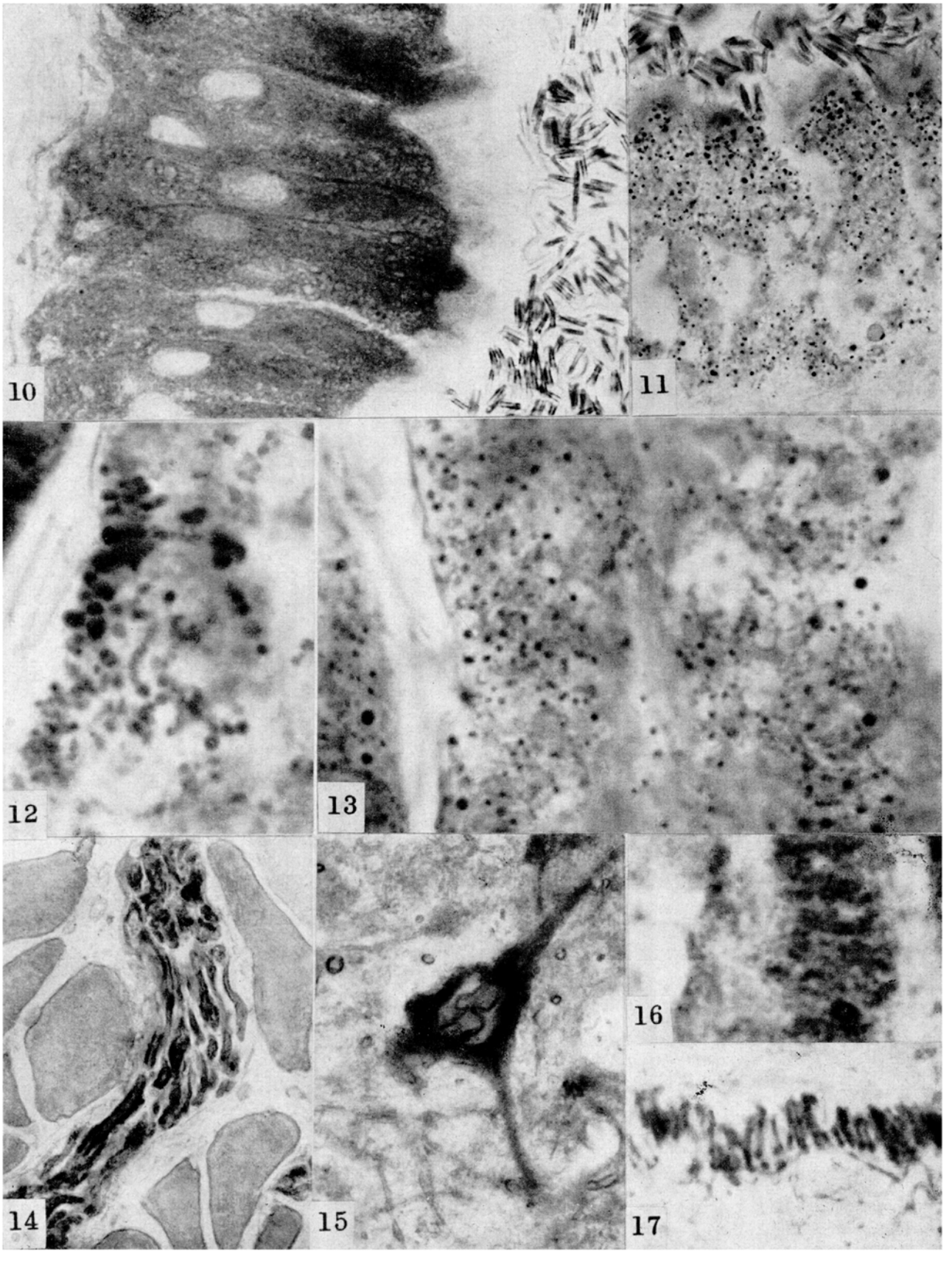

10
11
12
13
14
15
16
17

Fig. 10. Same reaction as Figure 7, but with Nitro-BT as an electron acceptor. Diffuse staining of cytoplasm, except fat vacuoles. Note staining of basement membrane. × 1250.

Fig. 11. β-Hydroxybutyrate dehydrogenase reaction using Nitro-BT. Incubation for 30 min results in crystallisation of uniformly deposited diformazan, mainly in the vicinity of fat vacuoles. × 1000.

Fig. 12. Formazan staining of protein absorption droplets in proximal tubular cells of rat kidney (18 h after intraperitoneal injection of ovalbumin). DPNH-diaphorase reaction on formalin-fixed tissue, using Nitro-BT. × 3000.

Fig. 13. Serial section to Figure 12. In contrast to other cytoplasmic sites, protein droplets are not stained by DPNH-diaphorase reaction when MTT-Co is used as an electron-acceptor. × 3000.

Fig. 14. Strong non-enzymic staining of myelinated nerve in rat pelvic muscle by reduction of adsorbed Nitro-BT. × 380.

Fig. 15. Diformazan staining of ganglionic cell and neuropil by non-enzymic reduction of adsorbed Nitro-BT. Formalin-fixed section of rat cerebral cortex. × 380.

Fig. 16. Diformazan staining of fragmented mitochondrial and membrane structures in a 2 μ cryostat section. Non-enzymic reduction of adsorbed Nitro-BT. × 2450.

Fig. 17. Serial section to Figure 16. Filamentous mitochondrial structures stained by Solochrome Cyanine R after postchroming by the Elftman procedure. × 2450.

Model experiments with various lipid fractions point to a multitude of tissue components which may be responsible for adsorbing tetrazole and it seems difficult to ascribe this property to a single chemical structure. It may be significant that all lipids which were shown to be capable of binding Nitro-BT contain unsaturated radicals. The composition of mitochondrial lipids is complex. Extracts of liver mitochondria for instance, contain cholesterol, sphingomyelin, lecithin and cephalin besides a large, undetermined fraction containing various lipid-soluble vitamins[21]. A great difficulty is encountered in the profound alteration which lipoprotein structures undergo in fixing and in the preparation of frozen-thawed sections. The status of lipid-protein interaction under such conditions is virtually unknown. We were unable to abolish the adsorption of Nitro-BT to sections previously subjected to extraction procedures for phospholipids[1].

Histologically, substantive tetrazoles are adsorbed by membranous lipoproteins structures. It is possible that the strongly substantive Nitro-BT forms a surface layer, the whole of which may be uniformly reduced along a given structure regardless of the site of the initial potential difference. Formazan staining of structures, adjacent to points of high reductase activity may be thus explained. In particular, this may explain the staining of structures like myelin which consist of multi-layered lipoprotein components[22]. Enzymic staining of whole myelinated nerve fibres by Nitro-BT in highly active muscle[23] and spinal cord[24] sections is likely to be an artifact.

A pattern basically different from that obtained by using substantive tetrazoles is formed by enzymically reduced MTT-Co. This particulate formazan does not stain any structure *per se* but appears to reveal the distribution of highly active electron-donating sites. Since formazan production has been shown to be a easumre of specific activity of enzyme particles[11] it can be assumed that dye produced at individual intracellular sites reflects the specific activity of these areas. The particulate structural element responsible for enzymic tetrazolium reduction can be both mitochondrial and microsomal. Alternatively it may not even belong to a morphologically defined entity, as in the case of bacterial cytoplasm[25]. The identical appearance of the particulate formazan, whether produced by suspensions of submitochondrial fractions or by microsomes, provides a reason for our previous failure to demonstrate extramitochondrial tetrazolium reductase[5,6]. It is evident, therefore, that a dot-like formazan pattern can no longer be considered to be due to mitochondrial activity only. In the animal cell, however, mitochondrial activity can be separated by using reactions for enzymes which are firmly structurally bound to mitochondria, namely succinic, β-hydroxybutyric and α-glycerophosphate dehydrogenase.

In enzyme histochemistry, one of whose aims is to characterise intracellular distribution of activity, it seems highly desirable to use non-substantive indicators or substrates giving rise to non-substantive products which, by means of efficient capture reactions, can reveal sites of enzymic activity without morphological bias induced by substantivity.

Zusammenfassung. Es wurden substantive Eigenschaften verschiedener Mono- und Ditetrazoliumsalze gegenüber Gewebebestandteilen im Schnittpräparat untersucht. Ditetrazole werden im allgemeinen stärker vom Gewebe adsorbiert als Monotetrazole. Ausnahmen bilden Monotetrazole mit polaren Substituenten oder Azobenzol-Resten in 3-Stellung. Ditetrazole mit elektronegativen Gruppen in 2-Stellung werden stark von Lipoprotein-Strukturen adsorbiert und zeigen in Modellexperimenten Substantivität für ungesättigte Lipoide. An verschiedenen Beispielen wird die Bedeutung substantiver Tetrazol-Eigenschaften für die Lokalisation von Dehydrogenase-aktivität im Schnittpräparat dargelegt. Die Lokalisation enzymatisch gebildeten Formazans ist im Falle von substantivem Tetrazol ein Ausdruck für die Adsorption der Farbstoffvorstufe an bestimmte Gewebekomponenten. Im Falle von nichtsubstantivem Monotetrazol besteht die Möglichkeit zur Bestimmung von Orten intrazellulärer Enzymaktivität ohne unspezifische Anfärbung von Gewebsbestandteilen. Die Bedeutung der stark voneinander abweichenden Eigenschaften von in der Histochemie gebräuchlichen Tetrazoliumsalzen für die Darstellung intra- und extramitochondrialer Aktivität wird diskutiert.

A. G. E. Pearse and R. Hess[26]

Department of Pathology, Postgraduate Medical School, London (England), November 29, 1960.

[21] R. D. Dallam and L. Shimomura, Arch. Biochem. Biophys. *79*, 187 (1959).

[22] J. B. Finean and J. D. Robertson, Brit. Med. Bull. *14*, 267 (1958).

[23] M. M. Nachlas, K. C. Tsou, E. DeSouza, C. S. Cheng, and A. M. Seligman, J. Histochem. Cytochem. *5*, 420 (1957).

[24] T. Samorajski, J. Neurochem. *5*, 349 (1960).

[25] R. Hess and F. M. Dietrich, J. biophys. biochem. Cytol. *8*, 546 (1960).

[26] *Acknowledgments.* We are indebted to Professor H. Beyer for the gift of MTT, to Dr. A. M. Seligman for Nitro-BT, to Dr. A. W. Nineham for M and B 1762 A and 1767 and to Dr. G. Glenner for BCT. We are grateful to Dr. G. Popják for assistance in the preparation of cell fractions. One of us (A. G. E. P.) is in receipt of a Grant in Aid from the Medical Research Council.

STUDIORUM PROGRESSUS

Die Beeinflussbarkeit der Tiegelschen Kontraktur durch Änderungen der extracellulären Calciumkonzentration

Als «Kontraktion» bezeichnet man den über eine Muskelfaser (bzw. einen Ganzmuskel) mit der Erregung sich rasch fortpflanzenden kurzzeitigen kontraktilen Prozess der Zuckung. Unter «Kontraktur» eines quergestreiften Muskels versteht man eine länger anhaltende, auf den Reizort beschränkte, nicht fortgeleitete (also ohne rhyth-