# Variations in Reduction of Tetrazolium Salts by Dehydrogenase Systems.*† (25854)

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For assay of dehydrogenase activity in homogenates the monotetrazolium salt, 2-*p*-nitrophenyl - 3 - *p* - iodophenyl - 5 - phenyl tetrazolium chloride (INT), has been used most extensively(1,2). For histochemical demonstration of dehydrogenase activity in frozen sections the ditetrazolium salts, 2, 2′,5,5′-tetraphenyl - 3,3′ - (3,3′ - dimethoxy - 4,4′ - biphenylene) ditetrazolium chloride (BT), 2, 2′,5,5′ - tetraphenyl - 3,3′ - (4,4′ - biphenylene) ditetrazolium chloride (NT), and more recently 2,2′ - di - *p* - nitrophenyl - 5,5′ - diphenyl - 3,3′ - (3,3′ - dimethoxy - 4,4′ - biphenylene) ditetrazolium chloride (Nitro-BT), have been used(3,4,5). Introduction of nitro groups into the N-2 phenyl rings of BT (4) conveyed the favorable properties of INT (1) related to its relatively high redox potential(6), and in addition provided a highly substantive, insoluble pigment favorable for demonstrating activity in intracellular organelles(5,7,8). However, on comparing INT and Nitro-BT in both tissue sections and homogenate preparations, certain differences suggested the need for a systematic study of the influence of electronegative groups in the N-3 phenyl ring of 2-*p*-nitrophenyl tetrazolium salts. The 8 monotetrazolium homologues whose preparation is given elsewhere (9), are listed in Table I along with compound IX, which differs from INT (compound III) in having a C-5 methyl group in place of the phenyl group. Nitro-BT (compound X in Table II) was also included.

*Materials and methods.* Three dehydrogenases (succinic dehydrogenase, SDH; lactic dehydrogenase, LDH; isocitric dehydrogenase, IDH) were tested with the tetrazolium salts (I to X). Rat liver was homogenized (0.1-2 mg/ml) and fresh frozen sections of the same livers were cut in the cryostat at 12 $\mu$. Concentrations of substrates, pH values and duration of incubation were identical in the experiments using both homogenates and sections. Phenazine methosulfate was used as an intermediate electron carrier between the dehydrogenases and the tetrazolium salts whenever homogenates were used. Otherwise, the incubation media used were similar to those described previously(2,5,7,8).

*Results.* The extent of enzymatic reduction by tissue homogenates of the 9 tetrazolium salts is compared to INT in Table II. Replacement of the iodo group of INT with either a hydrogen atom (I) or an electropositive methoxy group (II) resulted in a marked decrease in rate of reduction by all 3 dehydrogenase systems. A weakly electronegative group, COOH (VI) was only slightly better as an electron acceptor for SDH and IDH, but not for LDH. Although it was expected from electronic theory that chloro (IV) and bromo (V) substitutions would enhance reduction over that of INT, this was not the case. Cyano (VII) and Nitro (VIII) substitutions yielded compounds which were poorer electron acceptors than INT with SDH, equally good acceptors with IDH, and as good as INT (VII) or better than INT (VIII) with LDH. Similarly, Nitro-BT (X) was comparable to INT with LDH and IDH, but only half as good with SDH. The important influence of the C-5 phenyl group was shown by the marked decrease in enzymatic reduction when the phenyl of INT was replaced by a methyl group (IX).

The finding that Nitro-BT accepted electrons at half the rate observed with INT in assay of SDH in liver homogenates was not in agreement with the equally good reduction

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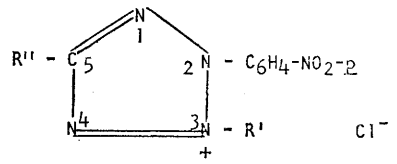
TABLE I. Tetrazolium Salt Homologues Tested.*




| Compound No. | R' | R'' |
|---|---|---|
| I | $C_6H_5$ | $C_6H_5$ |
| II | $C_6H_4$-$OCH_3$-*p* | " |
| III | " -I-*p* | " |
| IV | " -Cl-*p* | " |
| V | " -Br-*p* | " |
| VI | " -COOH-*p* | " |
| VII | " -CN-*p* | " |
| VIII | " -$NO_2$-*p* | " |
| IX | " -I-*p* | $CH_3$ |

* INT (III) was obtained commercially. The other tetrazolium salts were prepared in our laboratory(9).

of these 2 reagents in liver sections (Table II). On histochemical comparison of the 10 tetrazoles with the 3 dehydrogenases other discrepancies between the histochemical and biochemical behavior of these reagents was noted. For example, although Nitro-BT (X) is roughly similar to INT (III) with homogenate assays of LDH and IDH, Nitro-BT is a much better electron acceptor than INT in histochemical demonstration of LDH (Table II). A second nitro group (VIII) yields a tetrazole which is more readily reduced histochemically than INT by the 3 dehydrogenases, whereas in the homogenate assays INT is superior to VIII for SDH and comparable for IDH (Table II).

However, recent observations on sites of electron transfer in the succinoxidase system to several tetrazolium salts revealed differences that may help clarify some of these discrepancies(10). The loci of electron transfer in the respiratory chain were identified by use of purified soluble succinic dehydrogenase, inhibition by Antimycin A, removal of cytochrome c, inhibition with cyanide of cytochrome oxidase, and anaerobiosis. Homogenates were selected for study of the disrupted system, and tissue sections provided a means of testing the intact enzyme system. With the aid of phenazine methosulfate, electrons were passed from purified SDH to INT (2,10), in absence of phenazine methosulfate, electrons were passed to INT and Nitro-BT after cytochrome b but before the Antimycin A sensitive factor(10). The tetrazolium salts lacking nitro substituents (BT) received their electrons from reduced cytochrome oxidase (10). Explanations are not readily apparent for all differences in activity between the tetrazolium homologues noted in this study. Although the redox potentials of these tetrazoles are of some importance, it seems unlikely that this can be responsible for all the differences noted. The important issue which these data emphasize is that use of new tetrazolium salts may require prior investigation of sites of electron transfer as compared with those of INT and Nitro-BT, before safe interpretation of results can be made.

*Summary.* Electronegative groups attached to either the N-2 phenyl or the N-3 phenyl rings are important in determining the readiness of enzymatic transfer of electrons to tetrazolium salts. The magnitude of the effects exerted by nitro, chloro, or cyano moieties are not identical when different dehydrogenase systems are compared. Furthermore, differences noted between several tetrazolium salts in any one dehydrogenase system are not

TABLE II. Biochemical Reduction of Tetrazolium Salts by 3 Dehydrogenase Systems.

| Compound No. | SDH[a] | LDH[a] | IDH[a] |
|---|---|---|---|
| | (%) | | |
| I | 5 | 10 | 11 |
| II | 6 | 10 | 13 |
| III | 100 (2+) | 100 (2+) | 100 (2+) |
| IV | 47 | 62 | 78 |
| V | 65 | 64 | 50 |
| VI | 25 | 8 | 29 |
| VII | 76 | 100 | 98 |
| VIII | 40 (3+) | 117 (3+) | 94 (3+) |
| IX | 23 | 22 | 46 |
| X | 52 (2+) | 92 (4+) | 84 (2+) |

Compound No. refers to structures given in Table I. Compound X is Nitro-BT. Enzyme source was rat liver homogenate (2 mg/ml), and incubation media were similar to those described previously (2,7,8). Micromoles of formazan (or twice the micromoles of diformazan) produced when the 3 enzyme systems were tested with each tetrazolium salt were converted to per cent using values for INT (III) reduction as 100%. Averages of 3 or more experiments are given. Estimation of color density of histochemical preparations is given in parentheses on a scale of 1+ to 4+. Fresh frozen blocks of rat liver were sectioned at 12 $\mu$ and incubated for 15 min. at 37° in the reaction media described previously(5,7,8).

necessarily reproduced when biochemical and histochemical preparations are compared. Although the reasons for these differences are not immediately apparent, preliminary observations with the succinoxidase system suggest that some clarification may follow closer inspection of site of transfer to tetrazolium salts in the chain of electron transport.

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## Role of Local Trauma in Production of Cutaneous Calcinosis by Dihydrotachysterol.* (25855)

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A cutaneous calcinosis with sclerosis has been produced in rats treated during the first few days of life with parathyroid hormone(1) or with dihydrotachysterol, "DHT"(2), a Vit. D derivative that closely imitates parathyroid hormone in its pharmacologic properties. This skin lesion resembles human scleroderma in many respects. It has also been claimed that, in clinical scleroderma, calcium content of the skin is sometimes considerably increased(3,4) and that partial parathyroidectomy may induce marked improvement(5). Studies designed to clarify the possible interrelations between these clinical and experimental diseases would be greatly facilitated if a suitable laboratory model of this type were readily available. However, the previously described cutaneous lesions produced either with parathyroid hormone or with DHT do not lend themselves well to this kind of study because they: (a) are accompanied by high mortality, (b) develop only on the scalp and neck, (c) occur exclusively during the first few days of life and even then not in all instances. In the internal organs of DHT-treated rats, topical trauma can cause local calcification(6); clinical scleroderma also tends to develop at sites of local injury(7). The object of this communication is to show that, under suitable conditions, cutaneous calcinosis with sclerosis can be produced at will in predetermined regions of the skin, even in adult rats, if the selected area is lightly traumatized at a critical time of systemic DHT treatment.

*Methods.* For the sake of brevity, we shall not describe the many preliminary experiments necessary to establish optimum conditions for regular production of cutaneous calcinosis by DHT + local trauma. It should be emphasized, however, that DHT is most effective when given in oil, *per os,* and that predisposition for cutaneous calcinosis is greatest after very acute DHT intoxication. In one such experiment, 80 female Sprague-Dawley rats, mean body weight of 100 g (range: 85-115 g), were given 250 $\mu$g of DHT

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