to tumor incidence in the separate groups at the termination of each trial and lists the composite results of the three trials. These totals were obtained by combining the corresponding individual groups of each trial, thus giving a single "per cent tumor" value for the control mice and for each of the five cobaltinitrite groups. The effective total of mice in each group was, in most cases, less than the original number of 60. Obviously, this was due to deaths which occurred during the trial period. Of the 960 mice employed in all three phases of the experiment, only 28 died. Mice which died before tumors were noted in the group were not counted in the effective total. When a death occurred after growths had appeared, the animal was examined for the presence of a tumor, and the data obtained were incorporated into the final results. No evidence of cumulative toxicity was ever apparent throughout the experiment; the animals seemed normal in all respects. Table IV, which shows the initial and final weights of the various groups, fails to reveal any differences that might indicate some toxic manifestation.

Table III shows that of 176 control mice (those which received only the methylcholanthrene), 82 developed tumors of 1 mm. or greater in one dimension. This corresponds to a 47% tumor incidence, a result which is very close to the expected value of 50% and which reconfirms the MCD_{50} of methylcholanthrene. The mice which had received the two lower doses (30 and 40 mg./Kg.), responded to the MCD_{50} with a slight but insignificant decrease in tumors. However, at the 50-mg./Kg. dose level and higher, a significant reduction of tumor incidence was seen. Thus, bi-weekly, intraperitoneal injections of 50, 60, and 70 mg./Kg. of sodium cobaltinitrite were capable of reducing the per cent of tumor bearing mice to 31, 31, and 25, respectively, compared to the 47% control value.

It is difficult to accept that the reduction in tumor incidence is the result of the transient hypoxia produced by sodium cobaltinitrite. Periods of methemoglobinemia which lasted no longer than 3 hours and were elicited twice weekly certainly should not be unquestionably concluded as being the sole cause of this tumor inhibition. To reiterate the initial statements, the role of oxygen in the inception of the cancerization process is obscure, as is the basic mechanism of methylcholanthrene-induced carcinogenesis. It is quite possible that some metabolic effect other than hypoxia is responsible for or contributes to the decreased tumor incidence observed.

As a consequence of the theory that sulfhydryl enzymes may be involved in the induction of chemically-induced carcinogenesis by a fixation of the carcinogen to cellular proteins through sulfur linkages (10) an interesting concept arises. Lusky, *et al.* (11), obtained significant tumor reduction by applying dimercaprol (BAL) to mice during painting with 3,4-benzpyrene and reasoned that the additional SH-groups might compete with cellular SH for the carcinogen. Coupling this theory to the report by Vollmer and Carey (12)—that methemoglobinemia produced by sodium nitrite and *p*-amino propiophenone induces an increase in blood SH-enzyme levels—leads to the similar possibility that the increased SH-groups in the blood might also compete with the SH-groups of epidermal cell proteins, thereby affecting the neoplastic changes.

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Nonketol Reduction of Tetrazolium Salts in Pharmaceutical Analysis

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Nonketol compounds of pharmaceutical interest have been tested for reduction of tetrazolium salts; the quantitative applications of these reactions have been evaluated.

IN CONTRAST TO most organic compounds, the reduction of tetrazolium salts yields highly colored compounds. Based essentially upon this property, the production of these formazans by various reducing systems has led to a diverse range of biological and chemical applications. Lakon (1) has used triphenyltetrazolium chloride to detect viability of seeds, while Straus, Cheronis, and Straus (2) have applied tetrazolium salts in the study of carcinomatous tissue.

Chemical applications of tetrazolium salts include the quantitative analysis of reducing sugars (3), ketol steroids (4), and ascorbic acid (5). Rosenkrantz (6) has compared the effect of struc-

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tural changes on the reactivity of catecholamines with blue tetrazolium. Polyhydroxy phenols and readily oxidizable thiols have been determined qualitatively as a result of their ability to reduce tetrazolium salts (7).

To date, quantitative measurements involving tetrazolium salts have dealt primarily with the analysis of α-ketols. The objectives of this study were to determine the extent of nonketol reduction of tetrazolium salts and to evaluate quantitative applications in pharmaceutical analysis. Included in this investigation are polyhydroxy phenols, thiols, active hydrogen compounds, and other pharmaceutical reducing agents; that is, other compounds easily oxidized in the presence of air and light.

EXPERIMENTAL

Spectrophotometric measurements were made with a Beckman model DU spectrophotometer equipped with Bakelite thermospacers. Melting points are corrected.

Reagents.—*Blue Tetrazolium Reagent Solution.*—The 3,3'-(3,3'-dimethoxy-4,4'-biphenylene) bis[2,5-diphenyl-2H-tetrazolium chloride] (Dajac Laboratories) was purified by recrystallization from ethanol–anhydrous ether (8) until a portion of the salt in absolute ethanol showed constant absorbance at 254 mμ. Twenty-five milligrams of the purified salt was dissolved in 10 ml. of U.S.P. ethanol.

Red Tetrazolium Reagent Solution.—Twenty-five milligrams of 2,3,5-triphenyl-2H-tetrazolium chloride (Nutritional Biochemicals) was dissolved in 10 ml. of U.S.P. ethanol. (These solutions should be freshly prepared and protected from light.)

Tetramethylammonium Hydroxide Reagent Solution.—Ten milliliters of 10% aqueous solution was diluted to 100 ml. with absolute ethanol.

Test Compounds.—The compounds listed in Tables I–IV met the manufacturer's requirement for pharmaceutical use and were of official purity where applicable.

Screening Procedure.—Two milliliters of blue tetrazolium reagent solution followed by 2 ml. of tetramethylammonium hydroxide reagent solution were added to 10 mg. of test compound dissolved in 10 ml. of absolute ethanol. The reaction was allowed to proceed for 60 minutes at room temperature and quenched by the addition of 1 ml. of glacial acetic acid. The absorbance at 530 mμ was measured against a reagent blank.

Rate of Color Development.—From 10 to 400 mcg. of test compound was dissolved in 10 ml. of absolute ethanol and allowed to equilibrate in a constant temperature bath at 30.5 ± 0.1°. Two milliliters of blue tetrazolium reagent solution was added, followed by 2 ml. of tetramethylammonium hydroxide solution. The absorbance at specified time intervals compared to a reagent blank was measured at 530 mμ. Solutions were held in the thermostated (30.0 ± 0.1°) cell compartment of the spectrophotometer.

Assay Procedures.—An epinephrine standard of 10 mcg. in 10 ml. of absolute ethanol was prepared; a dilution of epinephrine solution (B.P.) of comparable strength in absolute ethanol was compared to this standard using blue tetrazolium reagent solution. The reaction was quenched after 40 minutes with glacial acetic acid.

About 80 mg., accurately weighed, of powdered 25-mg. diethylpropion hydrochloride tablets was suspended in 2 ml. of water and diluted to 250 ml. with absolute ethanol. A 10-ml. aliquot of filtered sample was compared to a diethylpropion hydrochloride standard of 200 mcg. in 10 ml. of absolute ethanol using blue tetrazolium reagent solution. The reaction was quenched as described above.

Table I.—Reactivity of Blue Tetrazolium with Polyhydroxy Phenols

	M.p., °C.	Absorptivity at Time, min. 10	30	60
Apomorphine HCl	[a]	2480	2490	2490
Epinephrine	210 dec.	5250	5240	5200
Hexylresorcinol[b]	64–65	328	416	438
Isoproterenol sulfate	119.5 to 122.5	3360	3380	3330
Levarterenol bitartrate[c]	97–101	2880	2950	2950
Protokylol HCl[d]	172–173	2460	2490	2450
Pyrogallol	133 to 134.5	3920	4140	4180
Resorcinol	109–110	133	378	661
Rutin[e]	206 to 209.5	340	617	830

[a] Oxidizes in air. Compounds generously supplied by: [b] Merck Sharp & Dohme. [c] Winthrop Laboratories. [d] Lakeside Laboratories. [e] Parke, Davis & Co.

Table II.—Reactivity of Blue Tetrazolium with Thiols

	M.p., °C. or (n^{25}_D)	Absorptivity at Time, min 10	30	60
l-Cysteine	224 dec.	1150	1330	1440
Dimercaprol	(1.5765)	1790	1960	2080
6-Mercaptopurine[a]	307–309 dec.	...	...	0.9
Methimazole[b]	144–145	...	...	7.8
dl-Penicillamine	196.5 dec.	612	634	643
Thiamylal[c]	131–133	...	...	0.2

Compounds generously supplied by: [a] Burroughs Wellcome & Co. [b] Eli Lilly & Co. [c] Parke, Davis & Co.

Table III.—Reactivity of Blue Tetrazolium with Other Pharmaceutical Reducing Agents

	M.p., °C. or (n^{25}_D)	Absorptivity at Time, min. 10	30	60
Anthralin[a]	173–176	783	1010	1100
Menadione	103.5 to 104.5	1440	1490	1500
Phytonadione[b]	(1.5254)	35.0	193	371
Thiamine HCl	247–249 dec.	7.6	28.0	68.6
d-α-Tocopheryl acetate[c]	(1.4924)	...	...	16.1

Compounds generously supplied by: [a] Abbott Laboratories. [b] Merck Sharp & Dohme. [c] R. P. Sherer.

RESULTS AND DISCUSSION

Nonketol pharmaceutical compounds were screened with blue tetrazolium for 60 minutes at room temperature. Only compounds which produced an absorptivity value over 20, based on the

TABLE IV.—REACTIVITY OF BLUE TETRAZOLIUM WITH ACTIVE HYDROGEN COMPOUNDS

	M.p., °C.	Absorptivity at Time, min. 10	30	60
Adiphenine HCl[c]	113–114	270	549	644
Bishydroxycoumarin[d]	285–287	...	...	0.7
Bromindione[e]	142–144	...	...	0.2
Cycloserine[f]	147 dec.	36.1	37.2	36.8
Diethylpropion HCl[g]	169–174 dec.	75.6	224	405
Dihyprylone[h]	101–103	...	...	1.0
Dimethindene maleate[c]	154 to 156.5 dec.	123	351	560
Diphenadione[i]	145–145.5	...	...	0.04
Ethotoin[d]	91–92	1230	1230	1210
Furazolidone[j]	268 dec.	665	1450	2000
Glutethimide[e]	81–85	...	...	0.01
Methantheline Br[k]	171–173	139	98.4	61.6
Methylphenidate HCl[e]	204–206 dec.	...	...	0.04[a]
Methyprylon[h]	74 to 76.5	...	...	18.6
Nitrofurantoin[j]	256–257 dec.	10.5	43.4	85.4
Nitrofurazone[j]	234.5 to 236.5 dec.	33.6	75.6	126
Phensuximide[l]	69–70	1180	1180	1170
Piperidolate HCl[m]	193–195	119	266	351
Potassium penicillin G[i]	211–215 dec.	...	...	10.4
Propantheline Br[k]	160 to 161.5	138	103	68.6
Santonin	172–174	...	...	0.1
Thiphenamil HCl[n]	125–127	462	477	470
Tolazoline HCl[e]	174–176	...	...	0.7
Valethamate Br[o]	122.5 to 124.5	...	...	0.02
Warfarin sodium[p]	160–162[b]	...	...	0.4

[a] First neutralized with tetramethylammonium hydroxide. [b] Melting point of Warfarin. Compounds generously supplied by: [c] Ciba Pharmaceutical Products, Inc. [d] Abbott Laboratories. [e] U. S. Vitamin & Pharmaceutical Corp. [f] Eli Lilly & Co. [g] Wm. S. Merrell Co. [h] Roche Laboratories. [i] Upjohn Co. [j] Eaton Laboratories, Inc. [k] G. D. Searle & Co. [l] Parke, Davis & Co. [m] Lakeside Laboratories, Inc. [n] Wm. P. Poythress & Co. [o] Ayerst Laboratories, Inc. [p] Endo Laboratories, Inc.

concentration of reducing agent, were considered sufficiently reactive to be of quantitative interest. Those compounds with an absorptivity over 20 were examined for rate of color development. Rates of reduction of blue tetrazolium by polyhydroxy phenols, thiols, and other easily oxidized compounds are summarized in Tables I–III by the absorptivity values at 10, 30, and 60 minutes. Also included are results for those compounds of low reactivity by listing the absorptivity at 60 minutes obtained from the screening procedure.

The reaction of triphenyltetrazolium chloride with alicyclic ketones (9) led to an extensive screening of

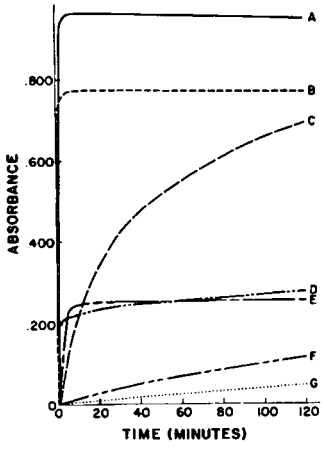

Fig. 1.—Rate of color development for 1×10^{-5} M solutions with blue tetrazolium. Key: A, epinephrine; B, apomorphine hydrochloride; C, rutin; D, dimercaprol; E, menadione; F, resorcinol; and G, thiamine hydrochloride.

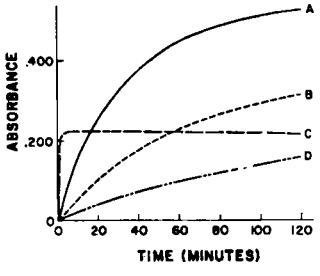

Fig. 2.—Rate of color development for 1×10^{-5} M solutions of active hydrogen compounds with blue tetrazolium. Key: A, furazolidone; B, dimethindene maleate; C, phensuximide; and D, diethylpropion hydrochloride.

active hydrogen compounds. Table IV summarizes the results of this study. Excluded from this table were 30 compounds in which the only activation of the hydrogen resulted from one or more aromatic rings. None of these compounds produced an absorptivity greater than 1.0 at 60 minutes.

Figures 1 and 2 are absorbance *versus* time graphs obtained for representative nonketol pharmaceutical compounds tested for rate of color development with blue tetrazolium. Figure 3 presents a comparison of the reduction of blue and red tetrazolium salts by ketol steroids determined under the same conditions as nonketols. The reaction of tetrazolium salts with steroids, exemplifying an established quantitative procedure, serves as a direct comparison to the compounds in Figs. 1 and 2.

Favorable comparison to the steroids concerning sensitivity of reaction and stability of color presents a strong indication of a successful assay for many compounds in Figs. 1 and 2 and others in Tables

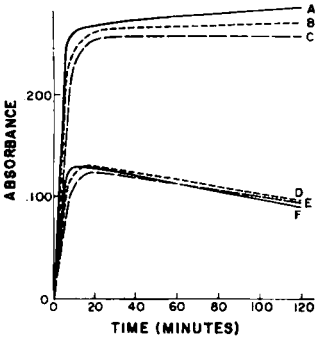

Fig. 3.—Rate of color development for 1×10^{-5} *M* solutions of ketol steroids with: I. blue tetrazolium—A, cortisone acetate; B, hydrocortisone; C, prednisolone acetate; and II. red tetrazolium—D, hydrocortisone; E, prednisolone acetate; F, cortisone acetate.

I–IV. For example, epinephrine (Fig. 1) exhibits greater sensitivity than steroids. The quantitative determination of epinephrine solution (B.P.) was highly satisfactory—ten analyses gave a recovery value of 97.54 ± 0.64% of labeled amount of epinephrine.

Diethylpropion hydrochloride (Fig. 2), in contrast to ketol steroids, represents a compound of lower sensitivity and does not plateau within 2 hours. Although this compound represents a less ideal example than epinephrine, ten analyses of 25-mg. diethylpropion hydrochloride tablets resulted in a satisfactory recovery of 99.24 ± 0.93% of labeled amount of diethylpropion hydrochloride.

A limited study was conducted using more vigorous reaction conditions to increase absorptivity values of compounds which were moderately reactive to blue tetrazolium. Rates of color development for methyprylon, *d*-α-tocopheryl acetate, and cycloserine were determined at 40°, other reaction conditions remaining unchanged. Methyprylon exhibited an absorptivity increase from 18.6 to 52.8 at 60 minutes, while *d*-α-tocopheryl acetate gave only a slight increase from 16.1 to 21.3. Cycloserine, which at 30° showed good stability of color but only moderate sensitivity, did not increase in sensitivity at 40°—and, in fact, produced a noticeable decrease in stability at this temperature. Although an increase in reaction temperature can have analytical significance in some cases, in limited examples it was of only minor importance and in the case of cycloserine was actually detrimental.

The reactivity of nonketol pharmaceutical compounds had been based exclusively on the reduction of blue tetrazolium. However, official monographs employing tetrazolium salts specify triphenyltetrazolium chloride. Rates of color development of several ketol and nonketol steroids were determined using red tetrazolium. Data for steroids are represented in Fig. 3, a comparison of the blue and red tetrazolium curves indicates greater color stability with blue tetrazolium. This superiority as well as the greater sensitivity of blue tetrazolium (4) was also exhibited with the nonketol reductions.

SUMMARY

Nonketol compounds have been tested for reduction of tetrazolium salts. Many compounds were reactive in low concentrations, indicating applicability to quantitative analysis in pharmaceutical formulations.

Specific procedures have been developed for epinephrine solution (B.P.) and diethylpropion hydrochloride tablets.

Nonketol compounds should be recognized as possible sources of interference to tetrazolium assays.

Comparison of blue and red tetrazolium salts using ketol and nonketol reductions indicates blue tetrazolium as a superior reagent.

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