REACTIONS OF CORTICOSTEROIDS WITH TETRAZOLIUM SALTS ON PAPER CHROMATOGRAMS

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INTRODUCTION

BUSH and coworkers[1] observed a 2.5-fold efficiency increase in the reduction of blue tetrazolium (BT) by corticosteroids on paper chromatograms by exposing the paper to one of the less polar solvent systems, in particular petrol ether. This fact, however, was attributed by BUSH to impurities of the reagent and no increase in efficiency was observed in the case of scrupulously pure BT. In our laboratory, methods for the estimation of corticosteroids using tetrazolium salts as spraying reagents have also been elaborated[2] and widely used. We were therefore very much interested in the action of non-polar solvents on the reduction of BT and some investigations have been made in this field. During this work some observations were made, leading to conclusions partially different from those reached by BUSH.

This paper reports some results of our investigations concerning the role of non-polar solvents on the reduction of BT and other tetrazolium salts and the chromatographic behaviour of formazans produced in the reaction.

EXPERIMENTAL

Corticosteroids

Authentic samples of cortisol, cortisone, corticosterone, Reichstein's compound S, and aldosterone were used for which we are indebted to CIBA A.G. (Basel), to N.V. Organon (Oss) and to the Research Institute for Medical Industry (Budapest).

Tetrazolium salts

These were prepared according to the usual methods[3]. The reagents were purified by paper chromatography in the Bush "B5" system (benzene–methanol–water, 2:1:1) and pure reagents were employed in the further investigations. The following tetrazolium salts were used:

2,3,5-Triphenyl-tetrazolium chloride (TTC); 2,3-diphenyl-5-*p*-tolyl-tetrazolium chloride; 2,2′-*p*-(di-*o*-methoxy)-diphenylene-3,3′,5,5′-tetraphenyl-ditetrazolium chlor-

ide (BT)*; 2,2'-*p*-(di-*o*-methoxy)-diphenylene-3,3'-diphenyl-5,5'-di-(*o*-nitrophenyl)-ditetrazolium chloride; 2,2'-*p*-(di-*o*-methoxy)-diphenylene-3,3'-diphenyl-5,5'-di-(*m*-nitrophenyl)-ditetrazolium chloride; 2,2'-*p*-(di-*o*-methoxy)-diphenylene-3,3'-diphenyl-5,5'-di-(*p*-nitrophenyl)-ditetrazolium chloride; 2,2'-*p*-(di-*o*-methoxy)-diphenylene-3,3'-diphenyl-5,5'-di-(*o*-hydroxyphenyl)-ditetrazolium chloride; 2,2'-*p*-(di-*o*-methoxy)-diphenylene-3,3'-diphenyl-5,5'-di-(*m*-hydroxyphenyl)-ditetrazolium chloride.

Chromatographic papers

Whatman No. 1. Whatman No. 2. Macherey-Nagel 214. Schleicher & Schüll 2043a. Schleicher & Schüll 2043b.

Solvents

All the solvents used were "pro analisi".

Method

Corticosteroids were dropped on to the chromatographic paper and run in the Bush "A" solvent system (ligroin–methanol–water; 10:8:2) for 4–12 h. The chromatograms were sprayed with the tetrazolium reagents (1 mg of the tetrazolium salt in 1 ml 5 % NaOH solution). After removing the excess of tetrazolium salt by washing with water, the papers were dried in air at room temperature. The formazans were eluted with ethyl acetate–methanol (7:3 v/v), the solution was made up to 3 ml and optical densities were measured using a Unicam SP 500 spectrophotometer.

Somewhat higher photometric values could be measured if the tetrazolium reaction was interrupted by acid, but the effect of non-polar solvent systems was the same in these cases too.

RESULTS AND DISCUSSION

Fig. 1 shows paper strips on which the formazan spots obtained by the reduction of BT with quantities of 10 μg** of cortisol (F), cortisone (E) and aldosterone (A) respectively are shown. Fig. 1a shows the untreated (control) and Fig. 1b the strips treated previously in the Bush "A" system. A significant increase in efficiency can be seen in the case of previous treatment with a non-polar solvent system in accordance with the quantitative measurements, as shown in Table I.

It is known from the literature[4] and from our own previous work, that on reduction of BT a mixture of formazan-like compounds is formed. Investigations have therefore been made on the chromatographic behaviour of the formazan produced after reaction with the reducing steroids in a non-polar solvent system. Paper strips treated as mentioned above were equilibrated with the Bush "B5" solvent system for 1 h then chromatographed for 45–120 min at 38°. Fig. 2 shows chromatograms obtained by this procedure. It can be seen that both on the untreated (a) and the treated (b) strips two main formazan fractions separate, one of them remaining at the starting point and the other moving along with the developing solvent front. Besides these two fractions a small fraction can also be observed in an intermediate position having a less definite R_F value. The quantity of formazans in these spots depends,

* Several other commercial BT samples including one from BDH were also used.

** The effect discussed here can also be observed in the case of very small quantities of steroids, but to illustrate this in the figure such high concentrations must be employed.

however, on the previous treatment. In the case of untreated paper strips, the quantity of deep blue coloured formazan remaining at the starting point is relatively small and a greater quantity appears at the solvent front which is also deep blue. In the case of the strips treated in the Bush "A" solvent system a greater quantity

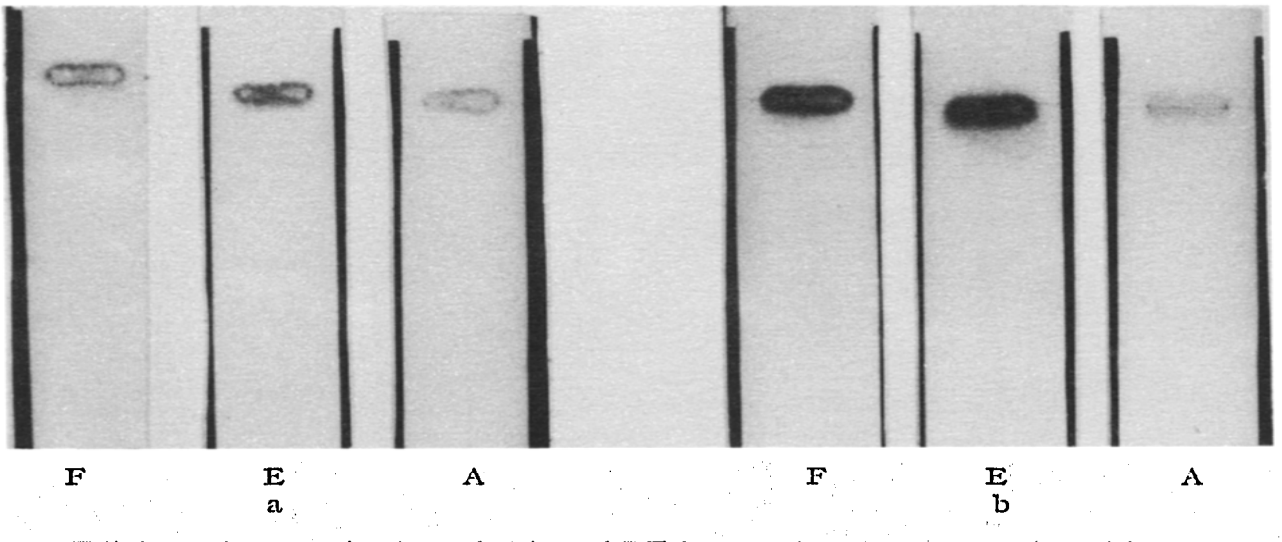

Fig. 1. Efficiency increase in the reduction of BT by treating the paper strips with a non-polar solvent system. (a) Control; (b) treated with the Bush "A" system.

of the more polar fraction remains at the starting point and a fraction of almost unchanged quantity moves along with the developing solvent front.

During investigations on the properties of formazan fractions mentioned above, it was established that the small red coloured fraction in the intermediate position could be easily removed by running the paper with 96 % alcohol just after the spray-

TABLE I

INCREASE IN EFFICIENCY IN THE REDUCTION OF BLUE TETRAZOLIUM BY TREATING THE PAPER STRIP WITH A NON-POLAR SOLVENT SYSTEM

Steroid 10 µg	*Optical densities of formazan solutions*		*Increase %*
	control	*treated*	
Cortisol	0.072	0.156	116
Cortisone	0.050	0.112	124
Aldosterone	0.056	0.112	100

ing of the chromatogram with BT and drying. The chromatogram of the formazan thus produced gave only the two deep blue coloured main fractions. The absence of the red coloured intermediate fraction could also be observed if the tetrazolium salt was dropped on to the chromatographic paper from alcoholic solution, the paper run in the Bush "B5" solvent system, sprayed with alkaline ascorbate solution and the formazan produced chromatographed again in the Bush "B5" system. Furthermore it could be observed that the quantity of the red fraction depended upon the purity

of the tetrazolium salts used. All these facts made it probable that the red coloured fraction in the intermediate position arose from impurities in the tetrazolium salts used and could be removed easily by running the paper with 96 % alcohol.

In addition to the chromatographic behaviour, the two main fractions showed

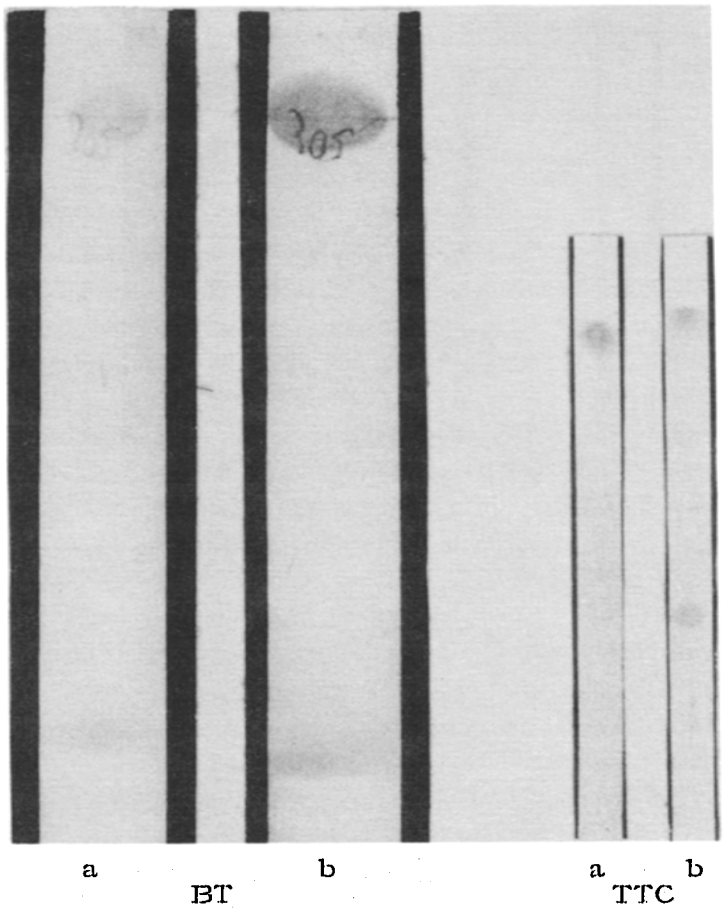

Fig. 2. Chromatograms of the formazans obtained by the reduction of BT and TTC by cortisol. (a) Control; (b) treated with the Bush "A" system.

other differences in their properties. The fraction remaining at the starting point could be dissolved rather slowly in common solvents (ethyl acetate, chloroform), while the fraction at the solvent front dissolved more readily. Absorption spectra of the two fractions were also different, as can be seen in Fig. 3. Moreover the fraction at the solvent front is more sensitive to light than the fraction remaining at the starting point.

These results made it clear that two definite and different substances are formed. The only question is whether they are derived from the same tetrazolium salt or not. To settle this problem both of these formazan fractions were oxidized to tetrazolium salts on the paper strips, then reduced with alkaline ascorbate solution or alkaline steroid solution and chromatographed in the Bush "B5" system. By this procedure, two formazan fractions, a "start" and a "front" fraction could be obtained again from both of the original fractions. This indicates that these fractions are not due to impurities in the tetrazolium salts but are obtained from pure tetra-

zolium salts under the conditions of reduction employed. Normally the so-called "front" fraction is the main product, while a non-polar solvent, such as petrol ether can change the ratio. It is noteworthy that enzymic reduction of blue tetrazolium in rat kidney homogenates *in vitro* gave only the "front" fraction.

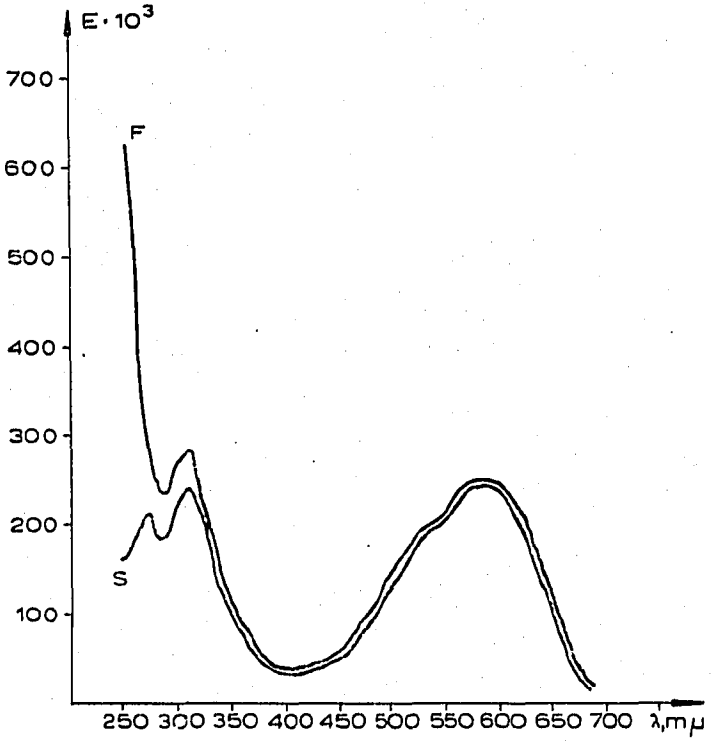

Fig. 3. Absorption spectra of the two formazan fractions in ethyl acetate–methanol (7:3 v/v) solution. S = fraction remaining at the starting point. F = fraction moving along with the solvent - front.

To obtain further information, an attempt was made to prepare these different fractions in crystalline form. Paper chromatograms on a preparative scale were developed and the formazan spots eluted with ethyl acetate–methanol (7:3 v/v). After removing the solvent *in vacuo*, a few milligrams of both of the "start" and "front" fractions were obtained. During this operation it was observed that the so-called "start" fraction is unstable both in solution and in the crystalline state, and upon rechromatography in the Bush "B5" system it seemed to be transformed into the "front" substance. On standing in air the "front" substance was partially transformed into a red coloured substance which could be easily dissolved in methanol and was not identical with the red coloured impurity mentioned above. It could be reduced with alkaline ascorbate solution to a blue formazan again. These transformations, however, could not be observed on the paper strips, only in the crystalline state or in solution.

Although the products of the reduction of ditetrazolium salts under various conditions form a rather complex system, we can say that this is certainly not due to impurities. On this point our opinion is different from that of BUSH. The two main fractions could be obtained not only by the reduction of BT, but also by that of other di- and monotetrazolium salts listed in this paper. The appearance of the two

fractions could not be explained either by the presence of partially reduced tetrazolium salts[5] especially when monotetrazolium salts had been employed. It seems probable that these two fractions are isomers of the same compound, one of them (the "start" fraction) showing a great tendency to transform into the other, which can be partially oxidized in air. The absorption spectra of the two fractions (Fig. 3) seem analogous to spectra obtained by HAUSSER and coworkers[6], who examined the "*cis-trans*" isomers of various formazans. A hypothetical reduction pattern of blue tetrazolium and other ditetrazolium salts can be deduced as follows:

$$\text{BT} \underset{\text{ox.}}{\overset{\text{red.}}{\rightleftharpoons}} \text{monoformazan} \underset{\text{ox.}}{\overset{\text{red.}}{\rightleftharpoons}} \text{diformazan ("front")} \rightleftharpoons \text{diformazan ("start")}$$

SUMMARY

Reduction of blue tetrazolium and other tetrazolium salts by corticosteroids on paper chromatograms gave rise to two formazan fractions, the amounts of which depended on the conditions employed. Evidence is presented that these fractions are not due to impurities. A hypothetical scheme for the reduction of blue tetrazolium and other ditetrazolium salts is given.

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