Color Test to Measure the Toxicity of Adrenal Cortex Hormones to Lymphocytes.* (18555)

ROBERT SCHREK.

From the Research Service, Veterans Administration Hospital, Hines, Ill., and Department of Pathology, Northwestern University Medical School, Chicago, Ill.

Two methods of studying the cytotoxicity of reagents have been presented in previous reports—the method of unstained cell counts (1) and a color test(2). The method of unstained cell counts is based on the property of living cells to resist staining with such dyes as safranine and eosin. By the addition of safranine, the number of living, *i.e.* safranine resistant, cells can be determined in a suspension before and after treatment with a reagent. This method was used to measure the toxicity of adrenal cortex hormones and other reagents. The color test is based on the capacity of a cellular suspension to reduce the oxidation-reduction indicator, 2,6 dichlorophenol indophenol, even when the suspension is exposed to air. The reduction of the dye was inhibited when the toxic agent, formaldehyde, was added to the suspension.

The question now arises whether the toxicity of adrenal cortex hormones to lymphocytes can be demonstrated and measured by means of the redox indicator.

Cytotoxicity of lipo-adrenal cortex. Cells of the rabbit thymus were suspended in a medium of serum and Ringer solution. The reagent, Lipo-Adrenal Cortex,† and the indicator, 2,6-dichlorophenol indophenol, were added to the suspension. The mixtures were incubated at 37°C in small test tubes which were shaken mechanically to prevent settling of the cells. In one hour the blue color of the test mixtures disappeared as a result of the reduction of the indicator. After about 7 hours of incubation, a slight blue color reappeared in the mixtures containing Lipo-Adrenal Cortex but not in the control suspensions without reagent. After 24 hours, the treated suspension had a distinctly blue color but the control suspension had only a trace of color. The differentiation in the intensity of color was sharp and definite. This experiment indicated that the Lipo-Adrenal Cortex had an inhibiting action on the reduction of the indicator by the cells. This inhibiting action was readily obtained with a low concentration of reagent (0.00,004 ml of the extract or 0.00,16 rat unit in the 0.4 ml of test solution). Furthermore, it was evident that the lipoid preparation had no immediate toxic effect but produced its first appreciable action after about 7 hours of incubation.

Titration of Lipo-Adrenal Cortex. The suitability of the redox test for the titration of adrenal cortex extracts and hormones was studied in a few preliminary experiments. A standard mixture was first prepared which consisted of equal parts of fresh and heat inactivated (56°C 1 hour) suspension plus a small amount of indicator. This mixture, evidently, contained one-half the number of living cells as the fresh suspension. The test preparations consisted of the fresh suspension with variable amounts of Lipo-Adrenal Cortex and with indicator. After 24 hours of incubation, the colors of the various mixtures were compared to determine which test preparation had the same intensity of color as the standard. This test preparation determined the titer of the reagent. In experiments on Lipo-Adrenal cortex, the suspension used contained 200,000 living cells per cubic milli-

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1. Schrek, Robert, *Endocrinology,* 1949, v45, 317.

2. Schrek, Robert, *Arch. Path.,* 1946, v42, 163.

† Lipo-Adrenal Cortex is a commercial preparation of The Upjohn Co. It is a purified and concentrated extract, in cottonseed oil, of the adrenal cortex. Each milliliter is reported to contain 40 rat units of cortical activity and to be equivalent to 1 mg of 17-hydroxycorticosterone according to the liver-glycogen deposition test.

TABLE I. Abstract of an Experiment to Determine the Titer of Lipo-Adrenal Cortex (Lac).

	Test mixtures				
Tube No.	Fresh-suspension, ml	Heated suspension, ml	Redox indicator 1:10000 ml	LAC rat units in .04 ml serum added to tubes	Intensity of color after incubation 37°, 24 hr
A	.32	0	.04	0	±
B	.16	.16	.04	0	++
C	0	.32	.04	0	+++
D	.32	0	.04	.0004	+
E	.32	0	.04	.0008	++
F	.32	0	.04	.0016	+++

Mixture in tube E had same intensity of color as tube B with 50% fresh suspension. The titer is, then, .0008 rat unit of lipo-adrenal cortex.

meter. The final concentration of indicator was 1:100,000. Under these conditions it was found that Lipo-Adrenal Cortex in a dilution of 1:20,000 produced a color equal to that obtained with a mixture of 50% fresh suspension. The titer of the commercial extract was then approximately .00,08 rat unit in the 0.4 ml of test fluid. The method used for the titration of the extract is exemplified in Table I.

The titration of the reagent has to be done with strict aseptic technic. The contamination of the suspensions with bacteria will usually cause the complete reduction of the dye and may cause the death of the lymphocytes. The color test appeared to be a simple and convenient method for the determination of the approximate titer of an adrenal cortex preparation. For a more accurate titer it would probably be necessary to use a pure adrenal cortex hormone as a standard and to use spectrophotometric methods to measure the color.

Cytotoxicity of other adrenal cortex steroids. Toxic effects, *i.e.* inhibition of the reduction of the indicator in the redox test, were obtained with 1 γ of cortisone acetate (Cortone, Merck). In contrast, 200 γ of desoxycorticosterone (Cortate, Schering) gave a negative test. The present findings with the color test and the previous observations by means of unstained cell counts are in accord. Both the color and the counting method showed that Lipo-Adrenal Cortex and Merck's Cortone are toxic but that desoxycorticosterone is not toxic to cellular suspensions derived from the rabbit thymus.

Specificity of the redox test. A positive redox test may be defined as an inhibition of the reduction of the indicator by a suspension treated with a reagent as compared to a control suspension without reagent. Positive tests were obtained with many reagents including formaldehyde, x-rays (100r) and nitrogen mustard (0.4 γ). Simultaneous viable cell counts indicated that these reagents in the doses tested were cytocidal to the lymphocytes. The color test was then not specific for adrenal cortex hormones but was positive with all of the toxic agents tested. As would be expected a positive redox reaction was also obtained with the oxidizing agent, potassium ferricyanide (0.001M). The reagent was, however, not toxic to the cells according to the method of unstained cell counts. Evidently a positive test does not by itself prove that a reagent is cytocidal. On the other hand, the reduction of the indicator by a cellular suspension does not necessarily indicate that the cells in the suspension are viable. The accidental presence of bacteria and other extraneous reducing agents in the suspension will decolorize the dye. In this connection, it may be noted that a cell in dying may liberate reducing agents. There may then be a lag time between the death of the cells and their loss of the ability of cells to reduce the dye.

These limitations in the use of the redox indicator as a measure of the number of viable cells does not invalidate the color test but does indicate the precautions needed in interpreting the results. Under the proper conditions, the color test provides a means of titrating the toxicity of extracts of the adrenal cortex.

Summary. Suspensions of living lympho-

cytes decolorize the oxidation-reduction indicator, 2,6 dichlorophenol indophenol. This reduction was inhibited by the cytotoxic agents, lipo-adrenal cortex, cortisone, nitrogen mustard and x-rays but was not affected by desoxycorticosterone. The method was used to titrate lipo-adrenal cortex, the titer being .00,08 rat unit. This color test seemed to be a convenient method for demonstrating and measuring the toxicity of the adrenal cortex hormones to lymphocytes.

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Action of Various Hormones on the Spread of Subcutaneously Injected Hemoglobin.* (18556)

PIERRE DUCOMMUN,† PAOLA S. TIMIRAS,‡ AND FRANCO DORDONI.§
(Introduced by Hans Selye)

From the Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal, Montréal, Canada.

The spread of a subcutaneously injected dye is dependent on numerous factors, the most important being Duran-Reynals' spreading factors(1). The hyaluronidase acting on hyaluronic acid in the mesenchymal ground substance is the best known but it is beyond doubt that apart from hyaluronidase, numerous enzymatic and non-enzymatic factors are involved in the spread mechanism. Hechter(2) has shown recently that spread is dependent upon various factors such as edema, trypsin, peptones and so forth. The mechanism of their action is not yet clarified. It seems to be either correlated with an increase of the local hyaluronidase secretion by the non-enzymatic injected spreading factor, or explained by alterations of non-hyaluronic components of the dermal barrier such as chondroitin sulfuric acid(3,4). Hormonal control of the spread was shown by Menkin (5) and Opsahl(6,7). They stated that adrenal secretions were related to the spread regulatory mechanism.

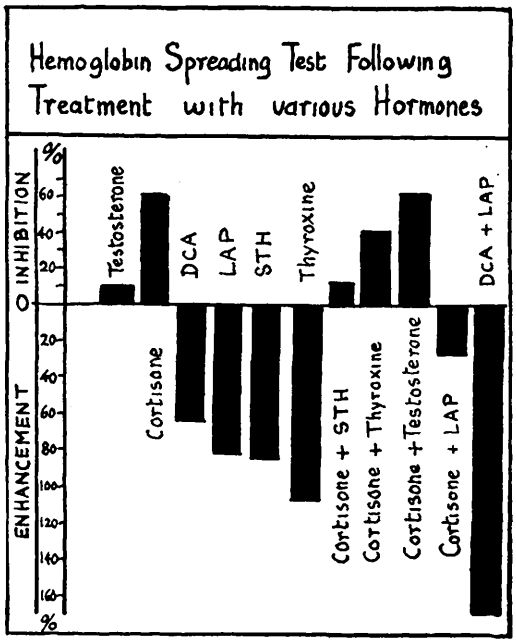

FIG. 1.

The purpose of this work was to control

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† Fellow of the Swiss Academy of Medicine and Biology.

‡ Fellow of the Canadian Life Insurance Officers Assn.

§ UNESCO Fellow (Canada).

1. Duran-Reynals, F., *Comp. rend. Soc. Biol.*, 1928, v99, 6.

2. Hechter, O., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1028.

3. Hobby, G. L., Dawson, M. H., Meyer, K., and Chaffee, E., *J. Exp. Med.*, 1941, v73, 109.

4. Duran-Reynals, F., *Bact. Rev.*, 1942, v6, 197.

5. Menkin, G., *Am. J. Physiol.*, 1940, v129, 691.

6. Opsahl, J., *Yale J. Biol. and Med.*, 1949, v21, 255.

7. Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1061.