hours will, upon release, cause, as has been thought by many investigators, a transfer of serum albumin into the tissue affected by the tourniquet. A comparison of buffered saline extracts of tissue from injured and uninjured legs by means of electrophoresis revealed large quantities of plasma albumin in the injured tissue extracts in contrast to only traces in the contralateral uninjured tissue extracts. The albumin was further identified by immunochemical procedures and by its sedimentation constant in the ultracentrifuge. The fact that the albumin electrophoretically-separated from the tissue extracts reacted quantitatively with an anti-dog albumin rabbit serum in the identical manner as the albumin electrophoretically-separated from the dog serum would seem to indicate that the albumin in the injured tissue came from the serum.

The effect of the tourniquet seems to lower but not remove the barrier between the blood and the tissue because, of the plasma proteins, only albumin, which is the smallest molecule with the highest negative net charge, is transferred to any appreciable degree. The electrophoretic analyses also indicate that the loss of serum albumin is localized to the injured area. It is believed that the small amount of albumin (Table II) appearing in extracts from the uninjured legs comes from residual plasma in the capillaries. Perfusion of the legs with saline after exsanguination resulted in leaving in the control only a trace of material with the mobility of albumin. These data, therefore, support the idea that plasma albumin goes exclusively into the injured tissues.

Summary Electrophoretic, ultracentrifugal and immunochemical analyses of a protein extracted in buffered saline from tissues injured by the application of a tight tourniquet to one hind leg of the dogs indicate that large quantities of serum albumin enter the injured tissue. However, little or none of the α- and β-globulins enters the tissue. The dye, T-1824, injected intravenously before release of the tourniquet appears in the tissue of the injured leg only.

Received June 26, 1951. P.S.E.B.M., 1951. v77.

Biologic Assay of Adrenal Cortex Hormones by the Method of Unstained Cell Counts.* (18901)

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Schrek(1,2) found that compounds B and F and extracts of the adrenal cortex had a direct cytocidal action on rabbit lymphocytes *in vitro*. Feldman(3) observed a direct toxic action on lymphocytes by compound A and by adrenal cortex extracts. In the present study, the lymphocytotoxic action is used for the biologic assay of the adrenal cortex hormones.

Methods. The reagents tested were compounds B, E and F, an extract of the adrenal cortex and chromatographic fractions of the extract. All of these reagents were kindly provided by Drs. William J. Haines and Marvin H. Kuizenga of the Upjohn Company. Various quantities of the reagents, dissolved in methyl alcohol, were distributed to small, sterile test tubes 100 x 13 mm. The alcohol was evaporated *in vacuo* at room temperature. The tubes with the dried reagent were stored

* Published with the approval of the Chief Medical Director, Veterans Administration. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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

2. Schrek, R., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 557.

3. Feldman, Joseph D., *Endocrinology*, 1950, v46, 552.

in the refrigerator. To prepare a cellular suspension, the thymus of a normal rabbit was removed aseptically. The tissue was chopped up with fine scissors in a medium of normal rabbit serum and Ringer's solution. The suspension was filtered through Monel metal wire screen.

The cellular suspension was distributed to the test tubes with the dried reagent. The tubes were incubated in a water bath at 37°C and were shaken during incubation at the rate of 200 times per minute to prevent settling of the cells. The number of viable cells was determined in the treated and untreated suspensions. First, a solution of safranine 1:4000 in Tyrode's solution was added to the suspensions. A drop of the mixture was placed in a hemocytometer and the number of unstained cells was counted. The cells which resisted staining were considered to be viable.

The original suspension usually contained about 100,000 viable cells per cubic millimeter. After incubation for 24 hours the number (C) of viable cells in the control suspension without reagent was approximately 50 to 70% of the original number. When the percentage survival was less than 50%, the experiment was not considered satisfactory and the data were discarded. In the suspension treated with reagent, the number of viable cells may be represented by R. The per cent effect was calculated by the formula

$$100\left(1-\frac{R}{C}\right).$$

The results obtained were represented in a probit or dose-effect curve (Fig. 1) with the abscissa, the logarithm of the dose of the reagent in the individual test tubes and the ordinates, the per cent effect in probit units. The dose producing a 50% effect was interpolated from the graph and was termed the cytocidal unit.

Compound F, E, and B. The dose-effect curve for compound F is curvilinear (Fig. 1) and appears to approach a maximum of 80%. Even large doses (200 γ) failed to kill all the cells in the suspension within 24 hours. A dose as small as 0.01 γ produced an appre-

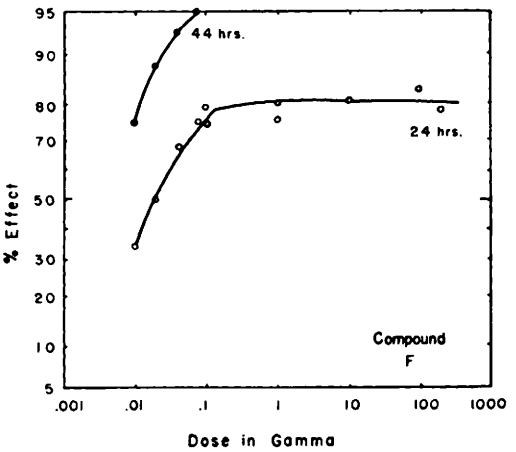

FIG. 1. Dose-effect curve showing % effect produced by 17-hydroxycorticosterone on rabbit lymphocytes incubated at 37°C for 24 and 44 hr. Abscissa shows log of the dose, and ordinates, the % effect in probit units. The % effect was calculated by the formula 100 (1–R/C) where No. of viable cells after incubation is represented by R for the treated and C for the control suspension.

ciable (34%) effect. The cytocidal unit or the dose that produced a 50% effect was, in this experiment, .02 γ.

The determinations of the cytocidal units of compound F in different experiments showed moderate variability (Table I). The arithmetic mean of the cytocidal units was 0.027 γ and the geometric mean, 0.024 γ. For convenience, 0.025 γ of compound F may be used as the standard cytocidal unit in the biologic assay of other hormones by the method of unstained cell counts.

In some experiments 44 hours of incubation was used as a period of incubation. The percentage of viable cells in the control suspen-

TABLE I. Frequency Distribution of Cytocidal Units of Compound F as Determined in Different Experiments.

Cytocidal unit (γ)	No. of cases
.010–.014	1
.015–.019	3
.020–.024	4
.025–.029	1
.030–.039	1
.040–.049	1
.050–.059	1
Total	12
Arithmetic mean	.027 γ
" standard deviation	.015 γ
Geometric mean	.024 γ
" standard deviation	1.65

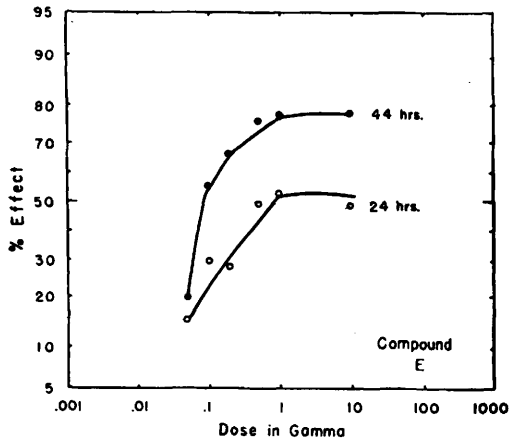

FIG. 2. The % effect produced by 17-hydroxy-11-dehydrocorticosterone on rabbit lymphocytes incubated at 37°C for 24 and 44 hr.

sion without reagent was 20 to 30% of that in the original unincubated suspension. The dose-effect curve in one experiment is shown in Fig. 1. At 44 hours, the per cent effect produced by any dosage of compound F was greater than at 24 hours of incubation. It appeared that the reagent had a slow and increasing effect on the cells. The 50% effect was produced by .016 γ.

The dose-effect curve for compound E incubated with cells for 24 hours (Fig. 2) is also curvilinear with a maximum effect of 50% in contrast to 80% for compound F. It is, therefore, not possible to determine for E cytocidal units based on the 50% effect in 24 hours.

The dose-effect curve after 44 hours of incubation showed a 78% effect with 10 γ of compound E, and a 50% effect with 0.1 γ. From these results, it is evident that compound F had both a greater and more rapid biologic effect than compound E.

The dose-effect curves for compound B incubated with cells for 24 and 44 hours were similar to those for compound E. The effect produced by 10 γ in 24 hours of incubation was 55% for compound B as compared to 50% for E. The 50% effect after 44 hours of incubation was .1 γ for both E and B. There was, then, no appreciable difference between the effects produced by compounds B and E.

Adrenal cortex extract. The dried adrenal cortex extract was supplied and titrated by Dr. Haines by means of the glycogen deposition method and was expressed in weight equivalents of compound F.

The cytocidal unit was found to be .08 γ —F equivalents as compared to .025 γ for compound F itself. Evidently the biologic assay of the adrenal cortex extract by the method of unstained cell count did not check exactly with that obtained by the glycogen

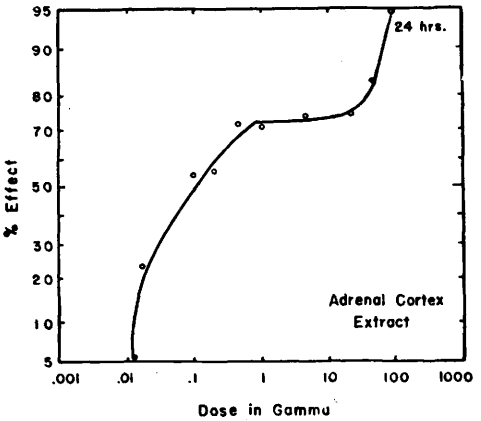

FIG. 3. The % effect produced by adrenal cortex extract on rabbit lymphocytes incubated at 37°C for 24 hr. Dose is expressed in wt equivalents of compound F as determined by the glycogen deposition method by Dr. Haines.

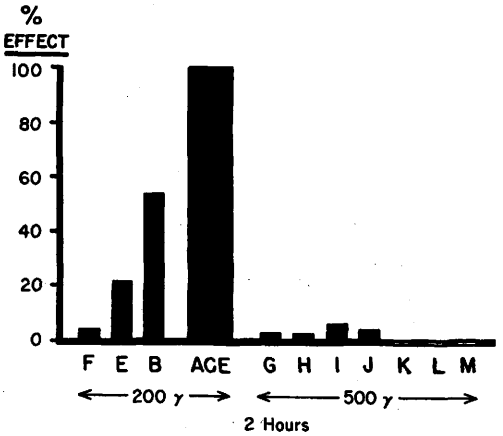

FIG. 4. The % effect produced by corticosteroids incubated with rabbit lymphocytes at 37°C for 2 hr. Preparations tested were 200 γ of the corticosteroids, F, E and B, 200 γ of adrenal cortex extract (ACE) and 500 γ of the chromatographic fractions, G to M of the adrenal cortex extract.

4. Feldman, Joseph D., *Yale J. Biol. and Med.*, 1950, v23, 114.

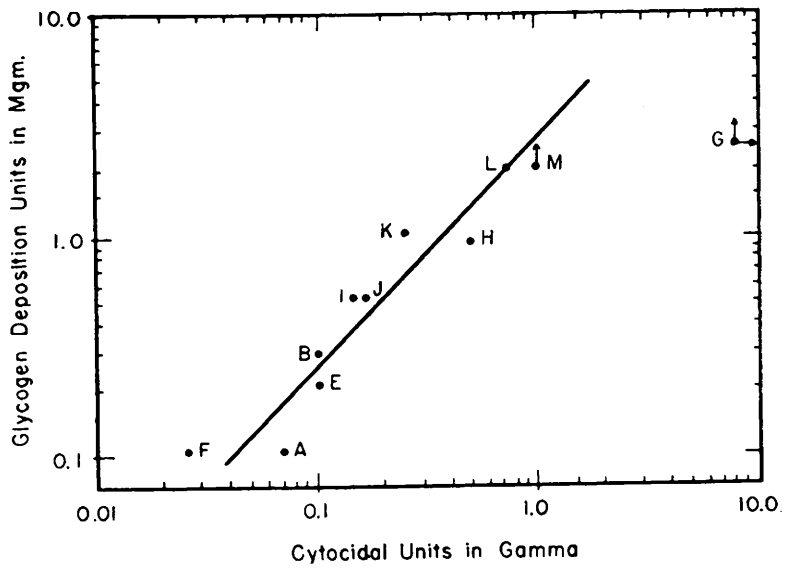

FIG. 5. Comparison of cytocidal units and glycogen deposition units for corticosteroids B, E and F, for adrenal cortex extract (A) and for chromatographic fractions G to M of the adrenal cortex extract. Cytocidal units were determined in this laboratory, and glycogen deposition units in the laboratory of the Upjohn Co.

deposition method. This is not surprising as the two methods test two different biologic properties of the extract.

The dose-effect curve for the extract (Fig. 3), is irregular in shape. With 10 γ —F equivalents of extract, the per cent effect was 75 which compared well with the 80% effect produced by 10 γ of compound F itself. With 100 γ —F equivalents of extract, there was a 95% effect although 100 γ of pure hormone F produced only an 80% effect.

In view of Feldman's report(3) that adrenal cortex extract had an immediate toxic effect on cells, and in view of the irregular dose-effect curve, a study was made of the toxicity of the extract after 2 hours of incubation at 37°C (Fig. 4). It was found that 200 γ —F equivalents of extract killed all the cells in 2 hours but 200 γ of compound F had no appreciable effect. Compound B did have a considerable effect (56%) and E a slight effect (22%). It may be concluded that not only the extract but also the pure hormones B and E in very large doses had rapid toxic effects on lymphocytes.

Comparison of two methods of biologic assay. Fig. 5 summarizes the findings obtained on the biologic assay of compounds B, E and F, the adrenal cortex extract (A in the figure) and of 7 chromatographic fractions of the extract (G to M). Dr. Haines prepared the chromatographic fractions and assayed the preparations by the glycogen deposition method. In this laboratory, the method of unstained cell counts was used for the biologic assay of the fractions.

According to both methods of assay, compound F was the most potent reagent tested with a cytocidal unit of .025 γ and a glycogen deposition unit of .1 mg. With the method of unstained cell counts and with 44 hours instead of 24 hours of incubation, compounds B and E had cytocidal units of 0.1 γ. With the glycogen deposition method, compound E was slightly more active with a unit of .2 mg as compared to .28 mg for compound B.

It is seen in the figure that the circles can be approximately represented by a straight line. Evidently, then, there is a fairly good correlation between the cytocidal unit and the glycogen unit.

Discussion. The mechanism of the delayed cytotoxic action of adrenal cortex hormones on the lymphocyte is not known. In view of the fact that compounds A, B, E and F, and not desoxycorticosterone affected by

lymphocytes, it would seem that the delayed lymphocytocidal action may be a function of the C-11-oxygenated hormones.

Feldman(3) demonstrated that large amounts of adrenal cortex extract had an immediate cytotoxic effect on lymphocytes. The present study confirms this finding. Feldman observed in addition that the extract, in large doses, killed not only lymphocytes but many other types of cells and that the immediate cytotoxic effect took place at room temperature as well as at physiologic temperatures. In this laboratory, small and moderate doses of adrenal cortex extract had no effect on cells other than lymphocytes and had no effect on lymphocytes at room temperature. In these respects the immediate and delayed cytocidal effect appeared distinct.

To explain the relationships between the immediate and the delayed cytotoxic effect of adrenal cortex extract, it may be assumed that a compound in the extract affects two distinct structures or processes of the cell. According to this hypothesis, small and moderate doses of the adrenal cortex extract alter a specific physiologic process in lymphocytes incubated at 37°C and hasten their death. A corollary of this hypothesis is that this physiologic process is absent or not essential to other cells at 37°C and to lymphocytes incubated at room temperature. In contrast, large doses apparently act as a universal protoplasmic poison and affect many types of cells at a wide temperature range.

A similar hypothesis may be used for the effect of x-rays on cells. Small doses of x-rays seem to have a specific action on mature lymphocytes and mitotic cells. Large doses affect all living cells. This "dual mechanism" hypothesis for the action of adrenal cortex and x-rays, seems useful as it explains a number of diverse findings.

Summary and conclusions. The proposed method of assay consists of incubating cellular suspensions derived from the rabbit thymus with adrenal cortex preparations at 37°C for 24 and 44 hours. The number of viable cells in the incubated and treated suspensions were determined by the method of unstained cell counts. The cytocidal unit was defined as that amount of reagent which killed 50% of the cells as compared to a control, incubated suspension. The cytocidal unit was found to be 0.025 γ for 17-hydroxycorticosterone and 0.1 γ for corticosterone and cortisone.

Small doses of the adrenal cortex preparations had a delayed cytocidal effect whereas large doses of adrenal cortex extract and corticosterone had an immediate effect.

Received June 26, 1951. P.S.E.B.M., 1951, v77.

Progressive Growth of C3H Mouse Lymphosarcoma in CF_1 Mice Treated with Cortisone Acetate. (18902)

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The relationships of growth and regression of strain specific transplantable mouse tumors when implanted in alien strains of mice has been established by Lewis(1). It was shown that spindle cell sarcomas arising in inbred strains of mice would grow progressively and kill when implanted in mice of the strain in which they originated. When transplanted in mice of alien genetic constitution however, the tumors would grow and then regress. The sojourn of such tumors for four days in alien strain mice conferred immunity against subsequent implantation of the same tumor.

Similar studies have recently been made by Kidd and Toolan(2) who implanted C3H mammary carcinoma and lymphosarcoma

1. Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1940, v67, 325.

2. Kidd, J. G., and Toolan, H. W., *Fed. Proc.*, 1949, v8, 360.