activated by DOC. These effects of DOC on cultured heart fragments probably do not represent any normal function of the steroid within the body, although Cleghorn *et al.* have indicated the possibility of a specific beneficial influence of DOCA on the heart. Adequate DOCA therapy will abolish the cardiac arrhythmia of adrenal insufficiency(8) although maintenance doses sufficient to return plasma electrolyte values to normal may not improve the electrocardiogram(3). The DOC-induced inhibition of the heart fragments in culture may be related to certain systemic effects of DOCA overdosage. DOCA and other steroids act as depressants(9,10). Weakness of skeletal muscle and irregularity of heart beat induced by DOCA can be prevented by increased potassium intake(11). In cultures the heart was less inhibited by DOC in the presence of potassium. Difficulties are likewise encountered in trying to relate cortical steroid effects in the body and on cultures of kidney, where cortisone increases excretion by mesonephric tubules(12).

Serum most probably interferes with the DOC effect by adsorbing the steroid(13) although the temporary recovery of hearts in the presence of an overdose of DOC points to a possible site of counteraction within the cell. If there is an antagonist in serum, it would not appear to be cortisone inasmuch as cortisone alone will not interfere with DOC, and sera presumably high in cortisone activity (placental or post-epinephrine) proved no more antagonistic than normal adult serum. Nor could any synergist in serum be demonstrated by blocking hearts in serum and then adding cortisone. The inertness of DOC acetate may result merely from its low solubility, but could also be interpreted as evidence that only the unesterified steroid is physiologically active and that heart tissue is unable to hydrolyse it.

Summary. DOC reversibly inhibits the pulsation of mouse or chick heart fragments *in vitro*. The inhibition can be removed by serum but not by cortisone, and is more effective in the absence of potassium. DOC and cortisone have no influence upon potassium inhibition of the heart. Epinephrine will almost completely counteract potassium inhibition but revives only a few DOC-poisoned fragments.

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Factors Influencing Measurement of Hyaluronidase Activity by Dermal Spread of an Indicator.* (18197)

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The enzymatic hydrolysis of the hyaluronic acid component of connective tissue ground substance as measured by the dermal spread

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of an indicator has become a useful tool for investigation. Employing this method of study, it became apparent that there was considerable difficulty in reproducing results. Well aware that there are many biological factors incapable of definitive control, this report presents the results of a study, in which controllable physical manipulations that are operating during an investigation of the enzymatic activity were varied. These factors include: the volume injected, the enzyme content, the rate of injection and the time elapsing between injection and measurement.

Materials and methods. Twenty-three young, female Sprague-Dawley white rats were used as the test animals. The hair was clipped closely over the entire dorsum and injections were made using comparable locations on each side of the midline for control and experimental studies. Aseptic technic was maintained throughout except that there was no preliminary sterilization of the injection sites. A commercial preparation of hyaluronidase† was employed which assayed 500 viscosity units per cc. Because the enzyme preparation contained sufficient sodium chloride to make it isotonic when dissolved in one cubic centimeter of distilled water, 2 preparations of the indicator substance, hemoglobin, were necessary. One, to be used as the control, was made isotonic with sodium chloride; the other, for solution of the enzyme, contained no sodium chloride.

Five experimental conditions were employed (Table I). The variable in each experiment is in italic print. The injections were made at a constant rate of injection, 0.01 cc/5 sec., except where the rate of injection was the variable. The area of spread was determined by caliper measurements of the length and width of the ellipsoid. The measurements were taken as rapidly as possible through the first 15 minutes of enzyme activity. The arithmetical means of area of spread for each minute were calculated. Measurements of continued spread were made at 5 minute intervals through 30 minutes, then every 10 minutes for the first hour. After that, the interval was every half hour, until the termination of the experiment at 5 hours. A total of 1056 observations was made during the course of the entire study, averaging 8 observations for each time period in each of the individual experiments. Seventy-one per cent of all single observations fell within a range of 10 sq. mm in area from the curve representing the arithmetical means of each experiment.

TABLE I.

Experiment	Vol. inj.	Enzyme content	Time of inj.
A	0.05 cc	0	25 sec.
B	0.05 cc	*25 units*	25 sec.
C	0.05 cc	25 "	*5 sec.*
D	0.05 cc	*12.5* "	25 sec.
E	*0.1 cc*	25 "	50 sec.

Results. Fig. 1 graphically presents the results of all the experiments. The ordinate is the logarithm of time in seconds and the abscissa is area in sq. mm. There is an overall pattern of spreading characterized by two phases of activity: the first, a period of very slow spread; and the second, characterized by an accelerated rate of spread. The variations in the spreading process produced by varying enzyme content, rate of injection, and volume of injection are clearly demonstrated. Fig. 2 is a presentation of the data from all 5 experiments on a single animal. It demonstrates the uniformity of results obtained. There are quantitative differences from the means obtained in the entire study, but qualitatively the general configuration of the curves in the two graphs is identical. It is evident, therefore, that each of the experimental variables, such as enzyme content, rate of injection and volume of injected material substantially influences the area of spread at a given time after injection.

A comparison of curve C with curve B of Fig. 1 shows that with constant volume of injection and constant enzyme concentration, the more rapid the rate of injection, the larger will be the area of spreading at comparable times. The area increase is con-

† Generously supplied as Alidase by Dr. Irwin C. Winter, Director of Clinical Research, G. D. Searle and Co., Chicago, Ill.

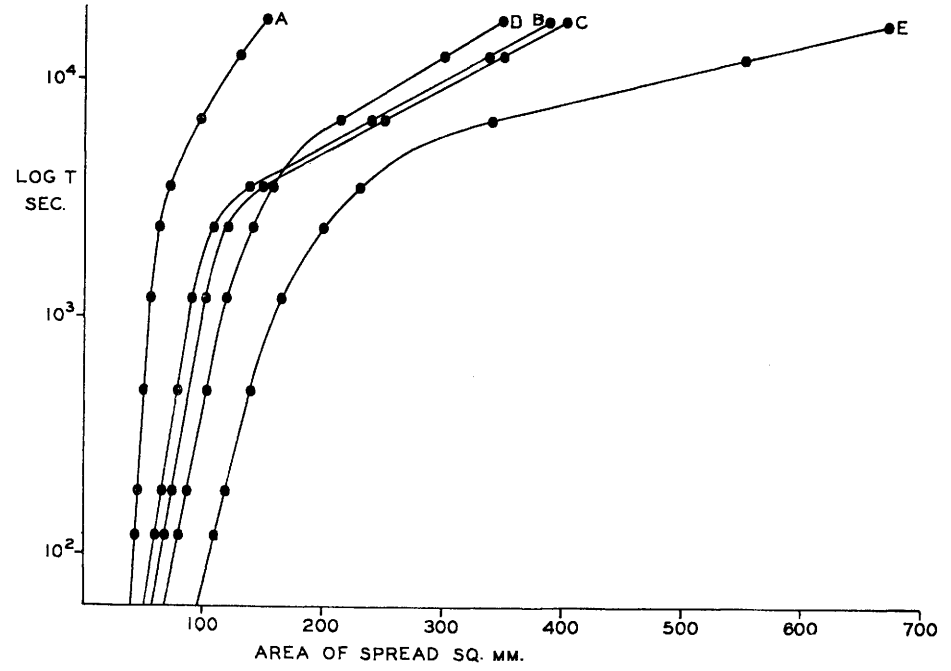

FIG. 1.

Variations in rate of spread resulting from altering certain factors: A—control; B—25 units enzyme; C—injection time 5 sec.; D—12.5 units enzyme; E—0.1 cc volume. (*cf.* Table I).

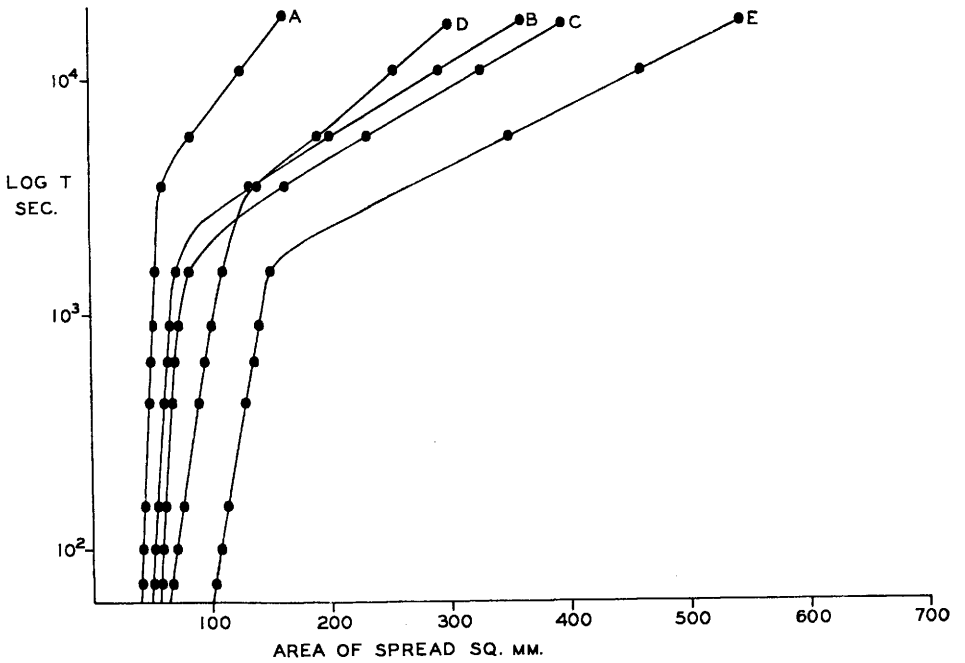

FIG. 2.

Graphic presentation of the data obtained from a single series of experiments on the same animal. (For explanation of the curves, see Table I).

sistently and uniformly greater throughout the 5 hour observation period. Curve D compared with curve B is remarkable in that with a constant rate of injection and constant volume, one-half the enzyme concentration produces a larger area of spread during the slow initial phase. The smaller area of spread in the rapid phase is the more to be expected result.

If the volume of the injected solution is doubled, but the enzyme content is constant and the rate of injection is unchanged, *i.e.*, .01 cc 5 sec. (curve E, Fig. 1), there is a much greater rate of spread compared with curve B, Fig. 1.

Discussion. In this study, no attempt was made to control age, weight, or state of the estrous cycle in the test animals. No evaluation was made of the individual animal's adrenal cortical response to the trauma of handling, operative manipulations and periods of restraint. Whether or not the regenerating hair pattern influences the dermal spread of hyaluronidase is not known and was not considered. Evans and Madinaveitea (1) have postulated on theoretical grounds that the kinetics of the spread of an indicator solution through the dermal layer might be governed by one or both of two steps. The first is a reaction between the diffusing factor molecules and the relatively nonpermeable wall of the dermal tissue. The second is the diffusion of the enzyme into the layer of solution which has been depleted of diffusing factor molecules by the reaction. The second step is supposed to be much slower than the first and is, therefore, the process which is followed experimentally. The data obtained in the present study fail to confirm such an interpretation since all the curves of activity are characterized by an initial phase of slow spread followed by a phase of more rapid spread.

The data from these experiments show that in employing the effect on the spread of an indicator by hyaluronidase as a biological measurement, certain physical factors must be carefully controlled. Failure to measure areas at uniform intervals after injection may result in considerable variation. The greatest error in reproducing comparable results arises from this single factor. For example, comparing curves A and B (Fig. 1), if A (the control) is read at 2 hours and B is read at 1½ hours, an error of 30 sq. mm (10%) obtains. Though the effect of rate of injection is consistently present, it constitutes a small error. From the shapes of curves B and C (Fig. 1), these appear to approach identical values after a period of 5 hours.

The peculiarity of a larger area of spread with a lower concentration of enzyme in the earlier phase of spreading as evidenced by curve D, Fig. 1, suggests that there may be an optimum concentration of enzyme for maximum spreading. Only when it reaches the more rapid phase does the curve conform to the usual pattern for the relationship of rate of reaction to enzyme concentration (2). Curve E, Fig. 1, illustrating the effect of increased volume of injection, shows a uniformly greater spreading effect, though the rate of injection and the enzyme content are constant.

It is suggested that the substrate concentration may be the limiting factor during the course of the slow phase of spreading. Such an interpretation would account for the experimental findings.

Summary. 1. The presence of several controllable sources of error in the study of indicator spreading by the enzyme hyaluronidase has been demonstrated. These are rate of injection, enzyme concentration, volume of the injection mass and measurement after equal intervals of enzyme activity. 2. Each factor of error singly or in any possible combination with others may materially alter the results of any investigation using this spreading as a biological measure.

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