CELL PENETRATION BY ACIDS.

VI. The Chloroacetic Acids.

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1. Method.

Several previous communications have provided measurements of the apparent rates of penetration of integumentary cells of the nudibranch *Chromodoris zebra* by different acids (Crozier, 1916 *a*, *b*). In the present report further data are given in continuance of this inquiry. The experiments were chiefly made in 1917. The work does not admit of very refined analysis, but inasmuch as the measurements are of use in related investigations (*cf.* Crozier, 1918 *b*), it is thought desirable to print them—particularly in view of the rare occurrence in animal tissues of indicators appropriate for such observations (Crozier, 1918 *c*).

Rather extensive dilution curves must be established for a number of acids, if the measurements are to be useful for discussion of the mechanism of penetration. Haas (1916) found that with solutions of external C_H. = 0.01 N, acetic acid penetrated various plant cells more quickly than did HCl, whereas several other acids, under these conditions, penetrated with equal speeds. The formal concentration of acetic acid at C_H. = 0.01 is about 4.4 N; of HCl, 0.01 N. If a similar comparison be made of two weak acids, acetic and butyric, it is found that in concentrations above 0.5 N acetic penetrates more quickly than does butyric (*cf.* Fig. 2), but in more dilute solutions the weaker acid, butyric, penetrates much more easily. At the point of intersection of the respective curves, the C_H. is higher with the acetic solution (2.55×10^{-3}) than with the butyric (2.1×10^{-3}). At the highest concentrations used, 1.0 N butyric penetrates in the same time

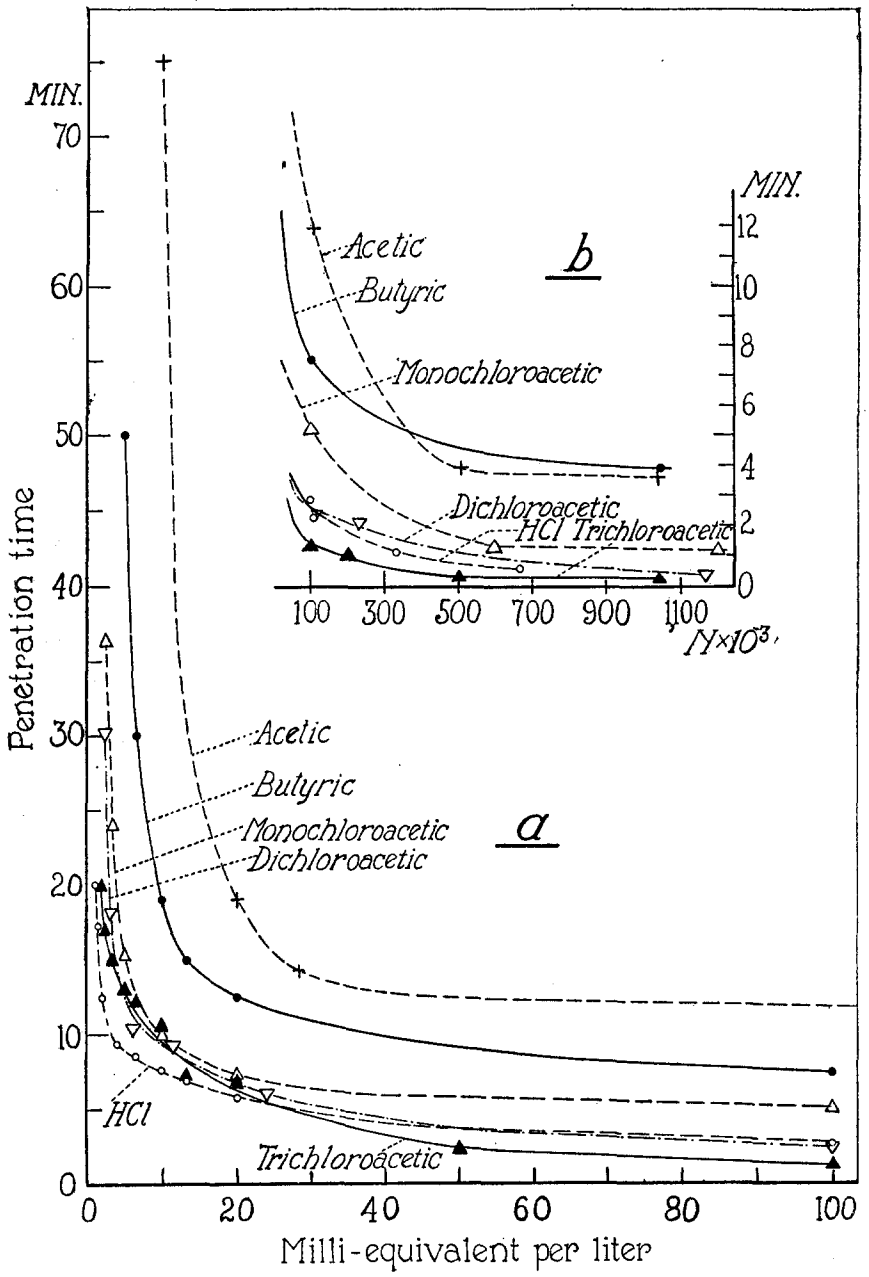

FIG. 1. *Abscissæ*, concentrations of acid, N×10−3; *ordinates*, penetration times, minutes. Each plotted point is the mean of a number of concordant readings. The zone of highest concentrations (*b*) and the lower range of concentrations (*a*) drawn separately, for clearness.

as 0.50 N acetic (3.9 min., at 27°), the C_H. being about the same in each case, 3×10^{-3} N. If comparisons were made only at this concentration, then, it might be stated that from solutions of equal pH these two acids penetrate cells within the same time; yet in all weaker solutions of corresponding pH values in the two instances, butyric penetrates more readily than does acetic (Fig. 1). These relations persist when a correction is applied taking into account the amount of acid required to produce the internal color change (Crozier, 1918 *b*). The relative effect of the hydrogen ion concentration on the speed of penetration is augmented with increasing concentration of acid; similar considerations apply to the comparison of HCl with acetic acid. Clearly, there are aspects of the penetration process, as observed in these experiments, which require for the analysis data pertaining to a range of concentrations.

Interpretation is facilitated, furthermore, by comparison of the acids within single chemical groups (Crozier, 1916 *a*). The chloro-acetic acids were therefore used, in conjunction with redeterminations of penetration curves for acetic and for hydrochloric acids.

The measurements plotted in Fig. 1 were secured according to the method outlined in an earlier paper (Crozier, 1916 *a*). It is necessary to again refer to certain aspects of the procedure.

The nudibranchs used were freshly collected individuals of nearly uniform size, and as nearly as possible of the same type of pigmentation. Strips were cut from the margin of the mantle-fold, which is conveniently erected as a result of handling the animal. Fig. 3, a cross-section in the mid-body region of a moderately contracted individual, indicates at 1.....1 the approximate line of cutting. From such strips pieces 1 cm. long were cut. A bit of mantle tissue so prepared is sketched at *B* of Figure 3, with its cross-section shown at *C*. Remaining in sea water for 10 to 15 minutes, the pieces were then wiped gently on filter paper, and placed in an acid solution. Pieces so prepared lived for a week in sea water. During several days they tend to show about the same resistance to the penetration of acid, but they more easily give up their intracellular pigment; the mantle is ciliated, and the tissue fragments therefore travel for some days over the bottom of the dish, so the vitality of a fragment is readily ascertained.

When a fragment of the mantle is placed in acid, a cut surface is presented for diffusion. It might be supposed that acid migrates through the spongy tissue laid open by the cutting, and that the observed color change depended upon this diffusion rather than upon penetration through the outer epithelial surface. I have already called attention to the fact that this idea would be erroneous (Crozier,

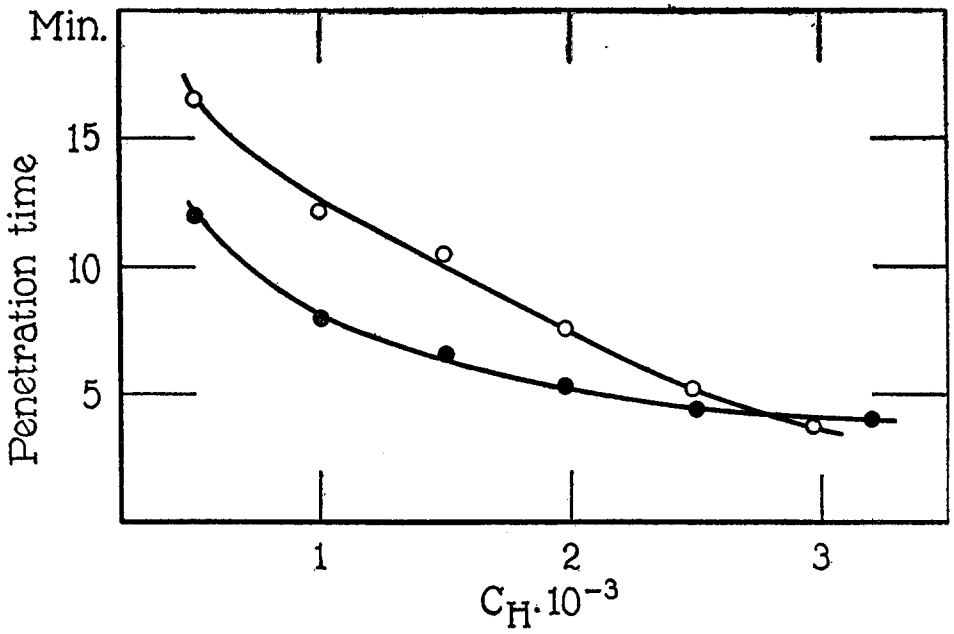

FIG. 2. Penetration of acetic acid (upper curve) and of butyric acid (lower curve) from solutions of corresponding pH.

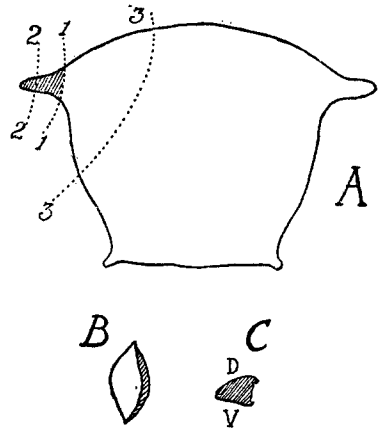

FIG. 3. *A*, Cross-section of *Chromodoris zebra* in the mid-body region; 1....1, line of amputation in preparing pieces of the mantle fold for penetration tests. Pieces removed at this depth give readings corresponding to those with pieces cut at the level 3....3, or with the entire nudibranch. Pieces removed at the level 2....2, present too great a proportion of damaged tissue, and penetration is very rapid in all cases. *B*, an isolated tissue fragment used in penetration tests, and *C*, its cross-section.

1916 *a*, page 263), but further experimental proof of this seems worth citing. Unless very small fragments of the tissue are used, 2 to 3 mm. broad, in which case there is usually a relatively considerable proportion of tissue mechanically injured in the zone of cutting, the penetration time of an acid solution is found to be independent of the size of the mantle fragment; it is identical with that obtained when the uncut whole nudibranch is immersed in the solution, provided the solution be stirred, and provided also that a sufficiently large volume of acid be used. The penetration, therefore, takes place through the outer surface of the mantle epithelium. The tests were made under such conditions—namely, with a large volume of solution—that the concentration of the external medium may be regarded as essentially constant. The results are therefore more simple to interpret than are findings such as those reported by Miss Hind (1916), where variations in the external concentration, only in part due to absorption of acid, form, in fact, the basis of measurement.

2. Relation to Size of Animal.

Mention has earlier been made of the fact that the penetrability of mantle tissue of *Chromodoris zebra* by acids is more rapid in the case of the smaller individuals (Crozier, 1916*a*). It should be important to decide whether this condition may have reference, primarily, to a decreased permeability of the outer surfaces of the cells in the older individuals, or rather to a general toughening of the protoplasm as a whole as age progresses. Particularly, it would be most desirable to secure a *measure* of the relationship between size, (age) and resistance to penetration.

Observations secured with HCl in a preliminary study of this matter are given in Fig. 4. The measurements, made in 1917, were carried out with due attention to such precautions as have previously been noted (Crozier, 1916 *a*; 1918 *b*). A few experiments with other acids (HNO_3, salicylic) showed that the greater apparent speed of penetration in the case of tissue from the smaller animals is quite general.

The regularity of the curves drawn invites their closer analysis. This cannot be carried very far, because in the existing data it is not possible to correct the observations according to the minimal concentration of acid which for each particular size of individual is required to effect the intracellular color change (Crozier, 1918 *b*); presumably, this correcting factor changes with increasing age. Nevertheless it is possible to arrive at a first approximation to a factor

giving a measure of the resistance offered by the tissue toward the inward diffusion of acid.

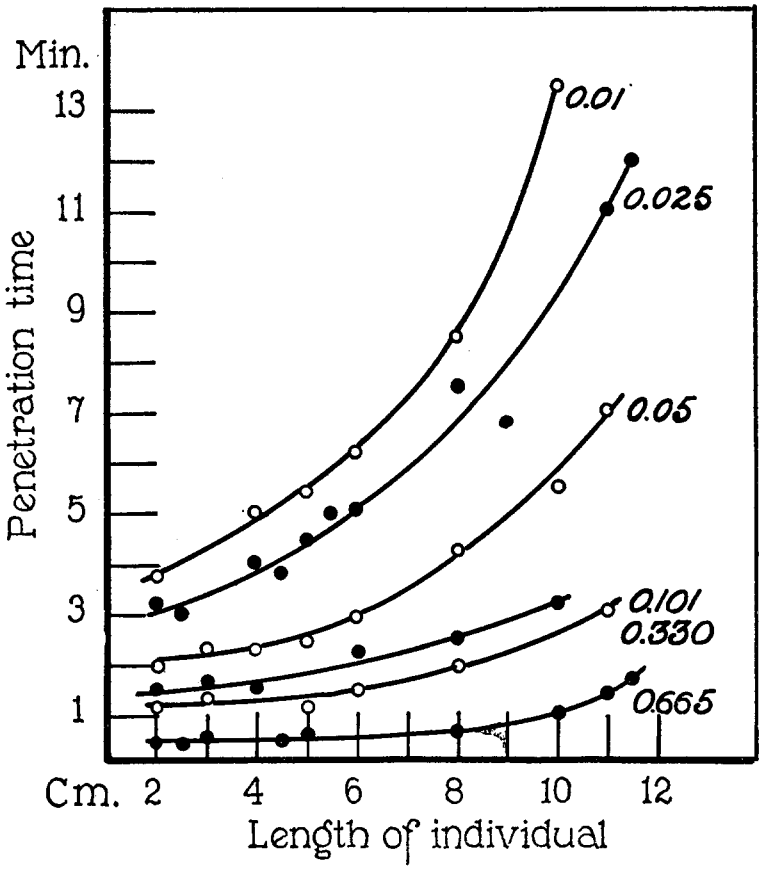

FIG. 4. Each curve shows the relation, for that concentration of HCl, between *observed time of penetration* (minutes), and *the size of individual* (cm.) from which the tissue had been obtained; each point represents the mean of a number of readings.

An equation has been suggested by Stiles (1920) which empirically satisfies the results of his experiments upon the diffusion of NaCl into agar gels containing $AgNO_3$. The equation has the form

$$\frac{p}{\sqrt{t}} = K \log C + K' \qquad (1)$$

in which t signifies the time elapsed for a distance of penetration p from a solution of concentration C; K' is a constant, varying chiefly with the nature of the penetrating salt; and K is a function chiefly of the concentration of $AgNO_3$ in the agar, constant for a given concentration of $AgNO_3$, but decreasing with an increase in this concentration.

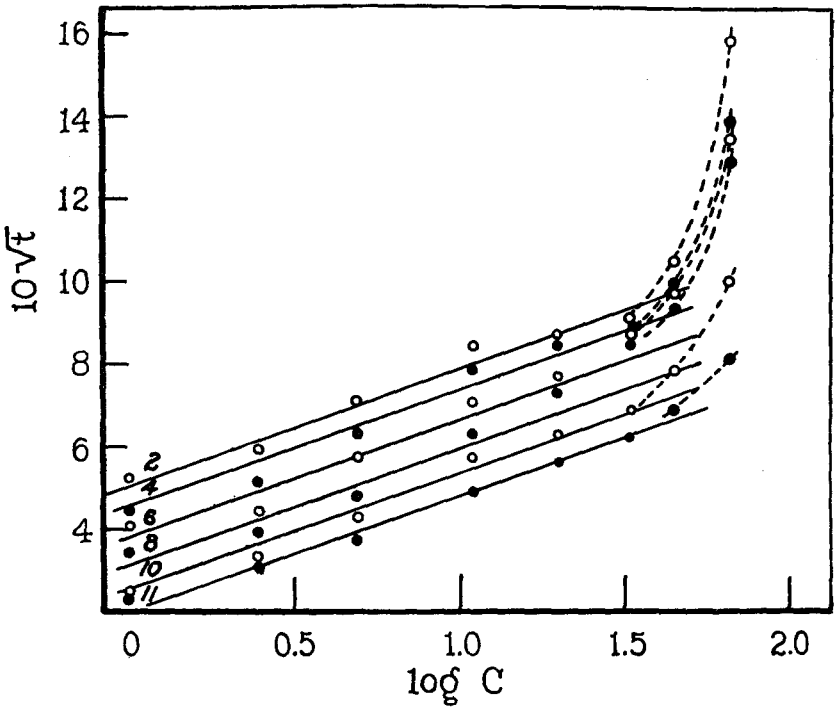

FIG. 5. To show that within a rather wide zone of concentrations the square root of the velocity of penetration (ordinate) is directly proportional to the logarithm of the concentration (abscissa); and that the graphs for the various sizes of individuals (2 to 11 cm.) are sensibly parallel within this zone of concentrations.

It may be assumed that in the *Chromodoris* tissue the distance p—to which there must have penetrated an amount of acid adequate to occasion the indicative color change—is essentially a constant quantity; Conklin (1912) has shown, in mollusks, that cell size is not a function of body size, but is constant during by far the greater part of the life duration. There is no evidence of age changes in the

distribution of the *Chromodoris* pigment. Under these circumstances, we have then

$$\frac{1}{\sqrt{t}} = k \log C + k' \qquad (2)$$

In Fig. 5 the observations already given are plotted in the form of equation (2). The points are taken from the smoother curves of Fig. 1. It is seen that the straight line equation (2) gives a fair

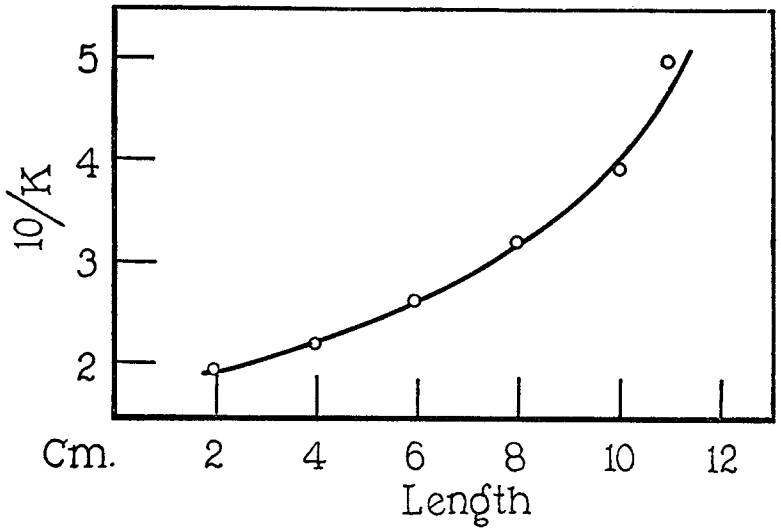

FIG. 6. Variation of $\frac{1}{k'}$ with size of individual.

account of the relation between size of individual and the penetration speed of each concentration of HCl. The deviations of the higher concentrations of acid from the straight line rule are due to the preponderating participation here of simple diffusion phenomena following rapid coagulation (Croizer, 1918 *b*), whereas with lower concentrations, the continuous combination of acid with protoplasm provides the slowest changes entering into the determination of the observed penetration time.

The straight lines in Fig. 5 are parallel, corresponding to the nature of the constant K in Stiles' experiments, whereas k' varies inversely

with the penetrability of the tissue. The reciprocal of k' may therefore be suggested as an appropriate measure of the relative resistance of the tissue toward diffusion of acid from the exterior. The magnitude of this reciprocal varies smoothly with the size of the individual, as shown in Fig. 6. Even taking into account the presumably higher "titration correction" applying to tissue from larger individuals, it may be argued from Fig. 6 that during growth the penetrability of the tissue decreases more rapidly than the length of animal increases.

Little can at present be said as to the physical meaning of $\frac{1}{K'}$. It is clear, however (from the analogy with the salt diffusion), that the resistance of the protoplasm as a whole increases with increasing size; data from growth studies in general suggests that this may in part be due to a diminution in the proportion of intracellular water. Conceivably, surface permeability varies in a parallel way. If this be the case, one factor in the lower excitability of larger individuals by acid solutions (Crozier, 1918 *a*) must reside in the tougher consistency of the superficial layer of the receptive elements.

3. Relation to Temperature.

The penetration of HCl from each of several concentrations was determined over a range of temperatures. The measurements are collected in Fig. 7. Solutions more dilute than N/160 were not employed, because with such solutions it would have been necessary to make a special study of correction, factors involving the minimal quantities of acid required at lower temperatures to effect color change in the tissue. In concentrations of HCl greater than N/100, these factors are relatively insignificant. Animals 8 to 11 cm. long were used throughout the experiments. Each of the plotted points (Fig. 7) represents the mean of several concordant measurements.

The values of the temperature coefficients exhibit some interesting variations. In Fig. 8, *temperature* is plotted against the *logarithm of the penetration velocity*. For the highest concentration used, 0.33 N, the graph is linear beyond 18°C., with Q_{10} constantly 1.89; for 0.101 N acid, $Q_{10} = 1.81$; 0.01 N, $Q_{10} = 1.68$; 0.0063 N, $Q_{10} = 1.19$. This series shows that with decreasing concentration some factor of

a purely physical character enters in a preponderating manner, especially below 0.01 N acid, into the determination of the observed result. The highest concentration provides a temperature coefficient of magnitude demonstrating that here some chemical event is probably the slowest in the series of changes resulting in observed penetration.

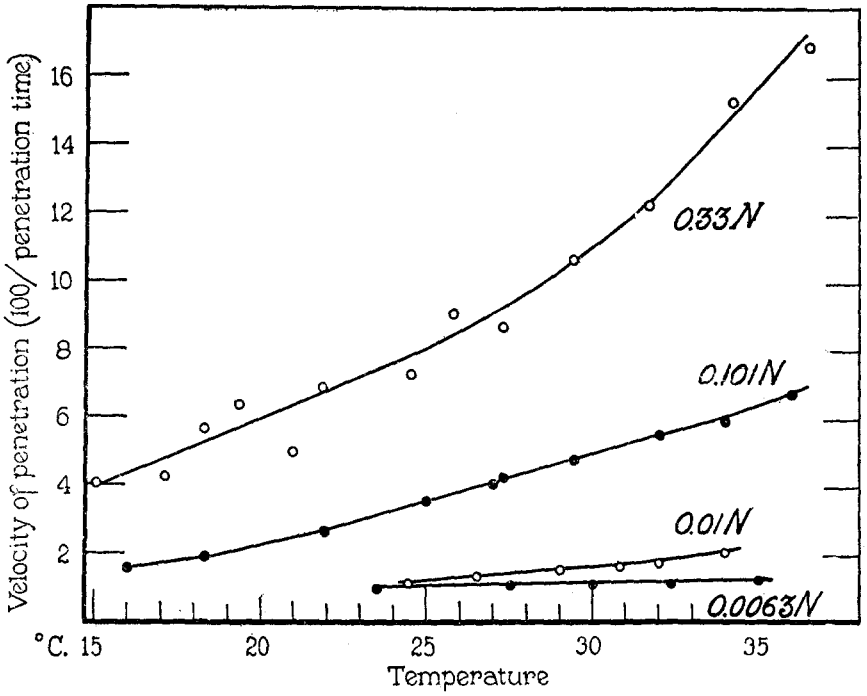

FIG. 7. Penetration of *Chromodoris* tissue by several concentrations of HCl, at different temperatures. The temperature range for normal activity of the whole animal is about 12–37°. Each curve concerns a single (indicated) concentration of HCl.

Osterhout (1914; 1917) has pointed out that the temperature coefficient of the electrical resistance of protoplasm is of the order 1.33, and that higher temperature coefficients obtained in different ways by other workers cannot be held to apply directly to the phenomenon of *permeability*. The present experiments are taken to signify that in the penetration of acid from dilute solution the observed velocity

of penetration is controlled chiefly by physical conditions having to do with the rate of diffusion, but that the initial event is a combination, of chemical nature, between acid and superficial protoplasm. This initial event is to be clearly observed only in rather concentrated solutions; its presence in more dilute solutions is inferential, but is strongly supported by the behavior of the chloroacetic acids, subsequently described.

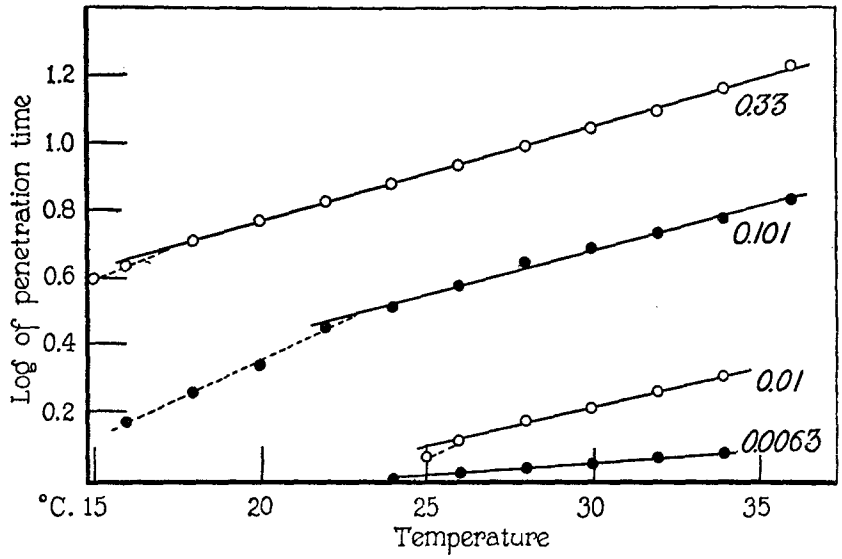

FIG. 8. As in Fig. 1, each curve pertains to a single concentration of HCl; points taken from smoothed curves in Fig. 1.

4. The Chloroacetic Acids.

The impossibility of establishing a seriation of acids, with reference to their relative penetrating ability, on the basis of observations at any single concentration, is sufficiently evident (*cf.* Fig. 1). Harvey (1914) found all three chloroacetic acids to penetrate tissue within about the same time, from 0.01 N solutions; this is also the case in my measurements, although the relative position of the three acids is further down in the list (at this concentration) than in the case of the tissue used by Harvey, and also (judging by the position of trichloroacetic) than for the plant cells used by Haas (1916).

Inspection of each penetration curve shows that beyond a certain ("critical") concentration the speed of penetration rapidly increases. The existence of a parallelism between the magnitudes of the respective concentrations at these critical points and the positions of the viscosity maxima in the protein-acid systems investigated by Pauli

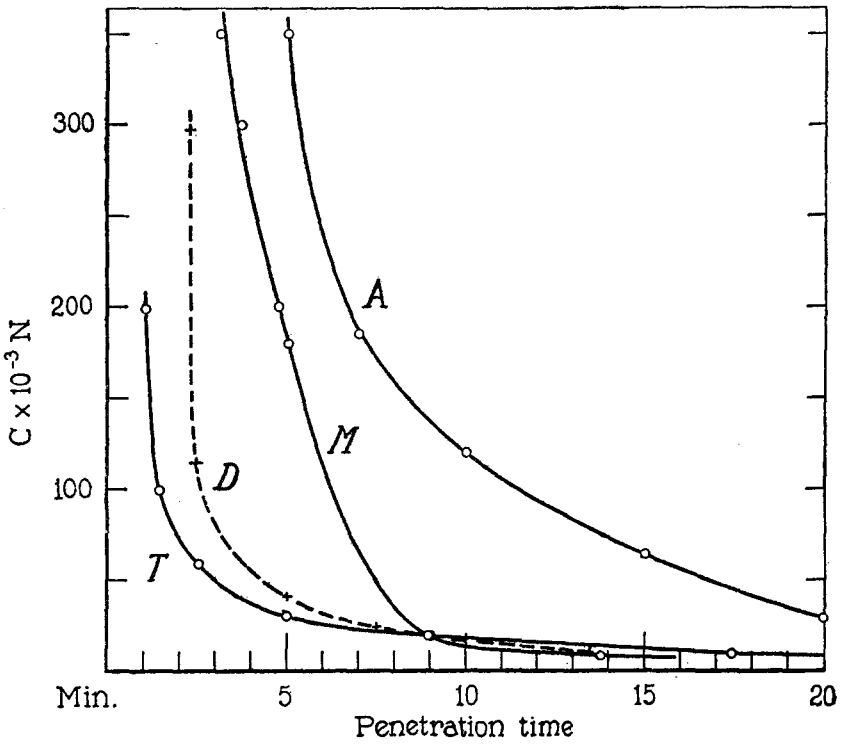

FIG. 9. Penetration time for acetic (*A*), monochloroacetic (*M*), dichloroacetic (*D*), and trichloroacetic (*T*) acids; concentrations and penetration times corrected according to the procedure described in the text. The observations extend beyond the limits of the figure. Points taken from smoothed curves in Fig. 1.

and Handovsky (1909), led to the idea that an effect upon cell proteins was primarily involved in penetration (Crozier, 1918 *b*). We now know, from Loeb's analysis of the chemical behavior of proteins (Loeb, 1920), that these viscosity maxima in fact occur at almost, if not quite exactly, the same pH in the case of all the acid-albumin

mixtures. The concentrations of the acids at the viscosity maxima state merely the relative quantities of these acids which at this pH are in equilibrium with the protein.

It may then be supposed that beyond some acid concentration corresponding to that involving a maximum viscosity of the tissue protein, the end-event in the penetration process becomes rapidly accelerated. The time intervals required to produce a maximum electrical resistance in *Laminaria* tissues immersed in a series of concentrations of HCl (Osterhout, 1914 *a*) show a good parallelism with penetration-time observations with *Chromodoris*. The velocity of penetration should in consequence depend both upon the strength of the acid and upon the readiness with which it is taken up by the protoplasm. The latter factor may vary independently of the acid strength; hence the desirability of comparing acids within groups of chemical relationship. It has been shown that in such groups, ionization determines the relative penetrating ability (Crozier, 1916 *a*),

The penetration curves for the acetic series obviously resemble, in their relative positions, the order and disposition of the curves showing the influence of these acids upon the C_H. in protein solutions (Loeb, 1920–21, page 90, Fig. 1). If it be that in order to secure corresponding speeds of penetration with different acids, a certain definite pH must be established by them in the superficial layer of the protoplasm, this constituting the "critical" event, then the members of the acetic series should arrange themselves in precisely this order. Choosing pH 3.5, corresponding, with all the acids, to about the acidity at maximum viscosity and maximum osmotic pressure (Loeb, 1920–21), as the probable critical pH, these acids should be equally efficient in the following relative concentrations (that of trichloroacetic taken as unity):

	Relative concentration.			
Acid.	Acetic.	Monochloroacetic.	Dichloroacetic.	Trichloroacetic.
pH				
4.0	7.6	1.16	1	1
3.5	10.2	1.12	1	1
3.0	26.0	1.67	1.04	1

To compare with such figures it is necessary to apply to the gross penetration data a correction which takes into account the quantity of each acid necessary to effect the intracellular titration (Crozier, 1918 *b*). This is done by subtracting from each nominal concentration the limiting concentration of that acid which is effective in penetration, and from the corresponding speed of penetration subtracting the speed of penetration of the limiting concentration. For the acetic series this has been done in Fig. 9; these curves show the ability of different concentrations of each acid to force into the tissue a quantity of that acid which is adequate to produce the indicative color change.

For comparison with the concentrations concerned in producing a definite pH in gelatin mixtures, we may choose a penetration time of about 7.5 minutes, as the absolute concentrations involved then correspond closely in the two cases (*cf.* Loeb, 1920–21):

	Relative concentration.			
Acid.	Acetic.	Monochloroacetic.	Dichloroacetic.	Trichloroacetic.
Corrected penetration time.				
5 min.	14.0	7.2	1.2	1
7.5 "	8.1	2.4	1.09	1

Qualitatively, a closer parallelism could not in the nature of the case be expected.

SUMMARY.

Measurements of the penetration of tissue from *Chromodoris zebra* are believed to show that a determining factor in penetration involves the establishment of a critical pH (near 3.5) in relation to superficial cell proteins. The rapidity with which this state is produced depends upon acid strength, and upon some property of the acid influencing the speed of absorption; hence it is necessary to compare acids within groups of chemical relationship.

The actual speed of penetration observed with any acid is dependent upon two influences: preliminary chemical combination with

the outer protoplasm, followed by diffusion. The variation of the temperature coefficient of penetration velocity with the concentration of acid, and the effect of size (age) of individual providing the tissue sample agree in demonstrating the significant part played by diffusion. In comparing different acids, however, their mode of chemical union with the protoplasm determines the general order of penetrating ability.

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