73. STUDIES ON DIFFUSING FACTORS
4. THE MODE OF ACTION OF DIFFUSING FACTORS

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The assay methods which have so far been used to measure the potency of diffusing factor preparations depend on the ability of these factors to increase the area of skin penetrated by a suitable indicator on intracutaneous injection. This

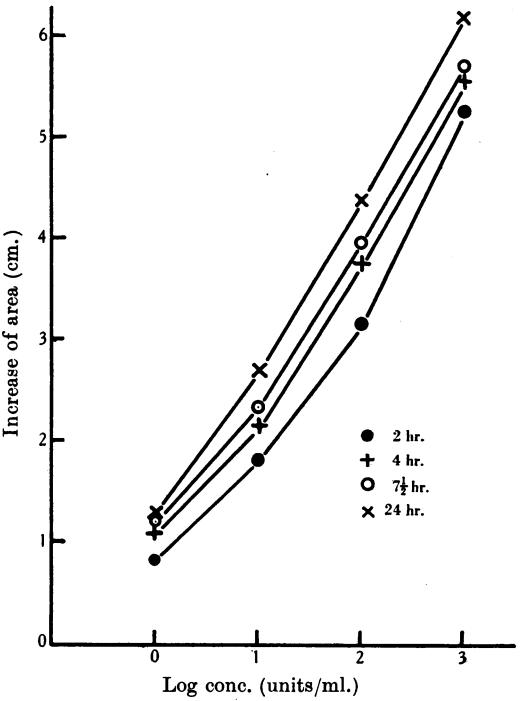

Fig. 1. Dose-response curve of testicular diffusing factor. Average of the results obtained in eighteen rabbits. Readings taken at different times.

increase is measured relative to a control injection without diffusing factor [Hofman & Duran-Reynals, 1931; McClean, 1930] or comparison is made with a standard preparation of diffusing factor [Madinaveitia, 1938]. In all these methods the margin of error is very large, and at best only consecutive tenfold dilutions can be differentiated with certainty. This very low accuracy is due rather to the remarkable flatness of the dose-response curve than to technical difficulties or individual variation in the experimental animals.

To establish the dose-response curve eighteen rabbits were used, four consecutive tenfold dilutions of the standard preparation of diffusing factor [Madinaveitia, 1938] being injected into the flanks of each animal. The solvent used was

an isotonic solution of haemoglobin and the experimental technique was that previously described [Madinaveitia, 1938]. The result is shown in Fig. 1, where, instead of representing the whole area penetrated by the haemoglobin as in previous papers, the increase in area relative to a control injection has been plotted; the figures obtained in this way are considered to represent the approximate net effect of the diffusing factor. Unless otherwise stated this method of representation is employed throughout this paper.

In Fig. 1 the average increase in the area of spread due to different concentrations of diffusing factor is plotted against the logarithm of the concentration. If the actual concentration were employed then with a scale of 1 cm. = 1 unit a strip of paper over 30 ft. in length would be necessary to represent the results. It seems at first sight astonishing that concentration should have so little effect on this manifestation of the action of diffusing factors. A possible explanation can be found if it be assumed that the active material present in the injected fluid is very rapidly eliminated or destroyed on coming into contact with the skin tissue. This assumption seems reasonable since it has been observed [Claude & Duran-Reynals, 1937] that intracutaneous injection of a concentrated solution of diffusing factor into the flank of a rabbit results after some time in the formation of a bleb of fluid in the abdominal skin of the animal, the injected fluid having fallen by gravity along the skin tissue and collected at its lowest point. The contents of such blebs were devoid of diffusing properties indicating that the tissues had destroyed or retained the active constituents. It is unlikely that they would be removed by the circulating blood since the action of diffusing factors is independent of circulation [Madinaveitia, 1939].

The work of McClean [1930] has shown that in the action of diffusing factors the spread takes place only in the true dermis. Working with coloured indicators he found that they penetrated neither the malphigian layer nor the epidermis.

The kinetics of the diffusing factor

The kinetics of the spreading of indicator solutions through the dermal layer might be governed by one or both of the following steps.

(*a*) A reaction between the diffusing factor molecules and the non-permeable wall of the dermal tissue.

(*b*) The diffusion of the molecules of "diffusing factor" into the layer of solution which has been depleted of diffusing factor molecules by the reaction.

Let us first consider the case in which this second step is much slower than the first and is therefore the process which is followed experimentally.

The law governing a diffusion process is

$$D\frac{\partial^2 c}{\partial x^2}=\frac{\partial c}{\partial t},$$

where c is the concentration of the diffusing substance, x the distance along the direction of diffusion, t the time and D the diffusion constant for the substance. If a is the thickness of the layers through which diffusion occurs, the differential equation for diffusion can be written (to within an accuracy of 0·4 %) in the integrated form

$$\frac{\bar{c}-c_0}{c_i-c_0}=1-\frac{8}{\pi^2}e^{-\frac{D\pi^2}{4a^2}t}, \text{ provided that } \frac{D\pi^2}{4a^2}t>0{\cdot}4$$

where $\bar{c}$ is the mean concentration of the diffusing substance in the layer of thickness a, c_0 the concentration of the diffusing substance in the layer at a time $t=0$ [Roughton, 1932] and c_i the concentration of the diffusing substance at the boundary of the layer; c_i is assumed to be constant.

In the experiments described here the diffusing substance, which may be the "diffusing factor" or the haemoglobin used as indicator, travels over a length of about 0·5 cm. in about 10 min. If then the concentration of the diffusing molecules is to remain constant over the whole layer the diffusion constant for the substance must be of the order of $D = 10^{-3}$–10^{-4}. This is a much higher diffusion constant than has been found for proteins, viz. $D \sim 10^{-7}$.

This diffusion expression can be used only if mechanical or thermal mixing effects are absent, and there is direct experimental evidence however that a slow diffusion process of molecules whose diffusion constants are comparable with those for proteins cannot be the rate-determining step.

If a layer of dermal tissue is treated with a solution containing the diffusing factor by injecting the solution and allowing it to spread to its full extent then it is found that a solution containing haemoglobin introduced into this pretreated layer spreads with great rapidity. Moreover, if a solution of diffusing factor containing haemoglobin as indicator is allowed to spread in the dermal layer the depth of colour is constant from the point of injection to the periphery of the spreading area at all times during the process. Since haemoglobin has a diffusion constant of 5×10^{-2} we should expect, if the diffusion occurs in an unmixed solution, a very marked change in the depth of colour, due to a steep gradient in concentration of haemoglobin, from the point of injection to the periphery of the area covered. That this is not the case would seem to indicate that mechanical or thermal mixing must occur to maintain a constant concentration of haemoglobin throughout the solution. Unless the diffusing factor molecules have a much larger diffusion constant than haemoglobin the same argument can be applied to the diffusing factor and we conclude that the liquid introduced into the dermal layer is mixed either by convection currents set up by the temperature difference between the two faces of the layer in which spreading occurs or by the mechanical effects of muscular action.

Reaction between the diffusing factor molecules and non-permeable wall

Let us now consider step (*a*), that is, reaction between the molecules of diffusing factor and the non-permeable wall of dermal tissue as the possible slow process.

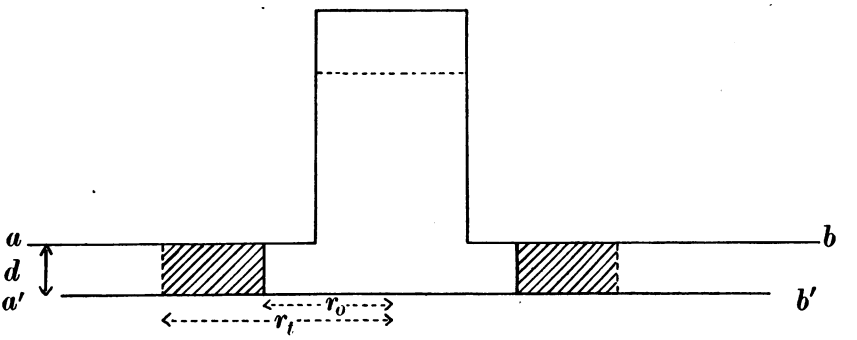

Fig. 2.

Fig. 2 gives a simplified model of the blister and the dermal layer which we shall use in this discussion.

The region between the two lines *ab* and *a'b'* represents the dermal layer of thickness *d* in which spreading is occurring, r_0 represents the radius of the blister

at time $t=0$ and r_t that at time t. The shaded area represents the region over which spreading has occurred in the time t.

Let us postulate that a reaction occurs between the molecules of diffusing factor and the non-permeable wall and that permeability is conferred upon the wall when unit area of wall of unimolecular thickness takes up n molecules of diffusing factor. This means that the non-permeable wall is pushed back by a thickness dr, the thickness of a unimolecular layer, and the radius over which "free diffusion" through permeable tissue can occur is now r_0+dr.

The rate-determining step is the rate at which the wall can be made permeable by the reaction between the molecules of diffusing factor and the wall.

The experiments of Claude & Duran-Reynals indicate that the reaction between the diffusing factor molecules and the wall is practically irreversible and hence we need consider only the velocity of the forward reaction, that is the rate of disappearance of the diffusing factor molecules, in considering the overall velocity of the spreading process.

We can treat this reaction as one between the molecules of the solute and a "solid" surface of variable area. The rate $\frac{\partial x}{\partial t}$ at which the solute molecules react with the surface is proportional to their concentration and to the area of surface exposed at any time.

This is expressed by

$$-\frac{\partial x}{\partial t}=kc.A, \qquad \text{.....(1)}$$

where c is the concentration of the diffusing factor molecules and A the area of the non-permeable wall exposed to the solution at any time.

The quantities $\frac{\partial x}{\partial t}$, c and A have the following explicit values

$$c=\frac{1}{V}(x_0-\rho d\pi (r^2-r_0^2))$$

where ρ is the number of diffusing factor molecules taken up per unit volume of dermal wall, and x_0 the number of diffusing factor molecules initially present in the solution. The volume V may be written

$$V=V_0+\alpha d\pi (r^2-r_0^2)$$

where V_0 is the initial volume of liquid injected and α is the fractional volume of water in the volume of tissue through which spreading has occurred which contributes to the spreading liquid.

The area of wall exposed at any time to the solution is $A=2\pi rd$, where r is the radius of the area covered and d the thickness of the dermal layer.

The rate of change of the number of diffusing factor molecules with time is

$$\frac{\partial x}{\partial t}=-\frac{\partial}{\partial t}\rho d\pi (r^2-r_0^2)=-\rho d\pi 2r\frac{\partial r}{\partial t}.$$

Equation (1) thus becomes

$$\frac{\partial r}{\partial t}=\frac{k[x_0-\rho d\pi (r^2-r_0^2)]}{\rho [V_0+\alpha d\pi (r^2-r_0^2)]},$$

thus giving an expression for the rate of change with time of the radius of the spreading area in terms of a reaction constant k, the number of spreading factor molecules initially present, the area covered and the initial volume. Equation (2), when integrated between the limits $r=r_0$ and $r=r$, gives

$$\frac{kt}{\rho}=\left[\tanh^{-1}r\sqrt{\frac{\rho d\pi}{(x_0+\rho d\pi r_0^2)}}\left\{\frac{V_0-\alpha d\pi r_0^2}{\sqrt{\rho d\pi (x_0+\rho d\pi r_0^2)}}+\frac{\alpha}{\rho}\sqrt{\frac{x_0+\rho d\pi r_0^2}{\rho d\pi}}\right\}-\frac{\alpha}{\rho}r\right]_{r=r_0}^{r=r}.$$

The volume of water contributed to the spreading solution from the volume of dermal tissue through which spreading has occurred is small compared with the volume of liquid injected. If α is so small that the volume $\alpha d\pi\ (r^2-r_0^2)$ can be neglected compared with V_0 the integrated expression reduces to

$$\frac{kt}{\rho}=\frac{V_0}{\sqrt{\rho d\pi\ (x_0+\rho d\pi r_0^2)}}\left\{\tanh^{-1} r\sqrt{\frac{\rho d\pi}{(x_0+\rho d\pi r_0^2)}}-\tanh^{-1} r_0\sqrt{\frac{\rho d\pi}{(x_0+\rho d\pi r_0^2)}}\right\}$$

writing $\pi r_0^2=A_0$ the area at time $t=0$ and $\pi r^2=A$ the area at any time t.

$$\frac{kt}{\rho}=\frac{V_0}{\sqrt{\rho d\pi\ (x_0+\rho dA_0)}}\left\{\tanh^{-1}\sqrt{\frac{\rho dA}{(x_0+\rho dA_0)}}-\tanh^{-1}\sqrt{\frac{\rho dA_0}{(x_0+\rho dA_0)}}\right\}$$

when the time reaches very large values $t\to\infty$, then the value of $\left(\frac{\rho dA_\infty}{x_0+\rho dA_0}\right)$ tends to unity, that is at $t=\infty$, then $x_0=\rho d\ (A_\infty-A_0)$ and the expression takes the simpler form

$$kt=\frac{V_0}{d\sqrt{\pi A_\infty}}\left\{\tanh^{-1}\sqrt{\frac{A}{A_\infty}}-\tanh^{-1}\sqrt{\frac{A_0}{A_\infty}}\right\}.$$

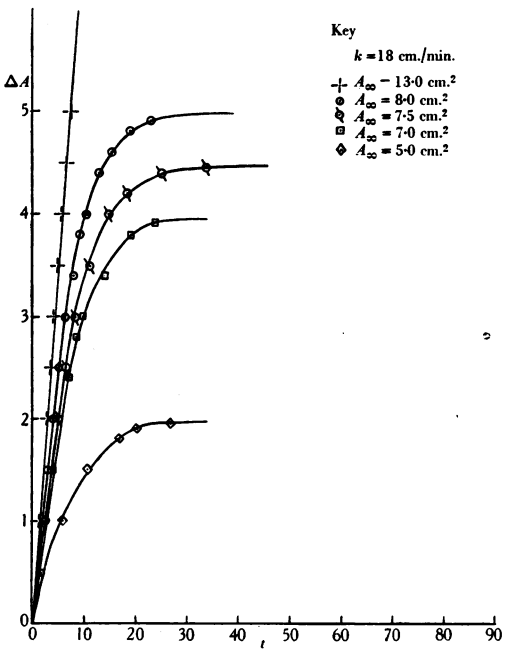

Fig. 3.

Figs. 3, 4 and 5 show the forms of the relation between area and time for various values of the constants in equation.

The important variables for this discussion are (i) the value of the velocity constant k, (ii) the number of diffusing factor molecules taken up per unit volume of dermal wall and (iii) the initial concentration of diffusing factor molecules in the solution.

Figs. 3 and 4 show the influence of change of x_0 and change of k respectively on the rate of spreading when ρ is large. Fig. 5 shows the influence of changes in x_0 and in k when ρ is small. One notices that the kallikrein behaviour is given

when k and ρ are small, whereas large values of these quantities lead to the diffusing factor behaviour. Fig. 6 shows both the experimental points for the increase in area under the influence of diffusing factor and kallikrein and in the

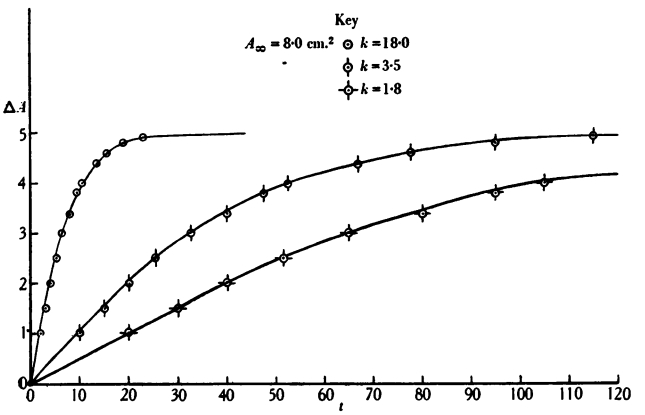

Fig. 4.

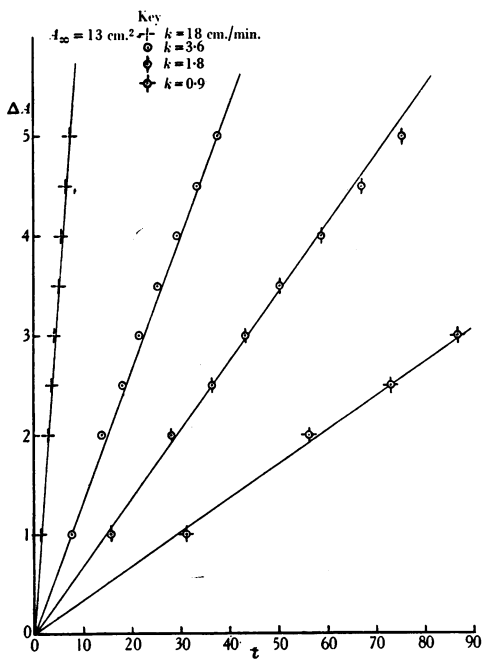

Fig. 5.

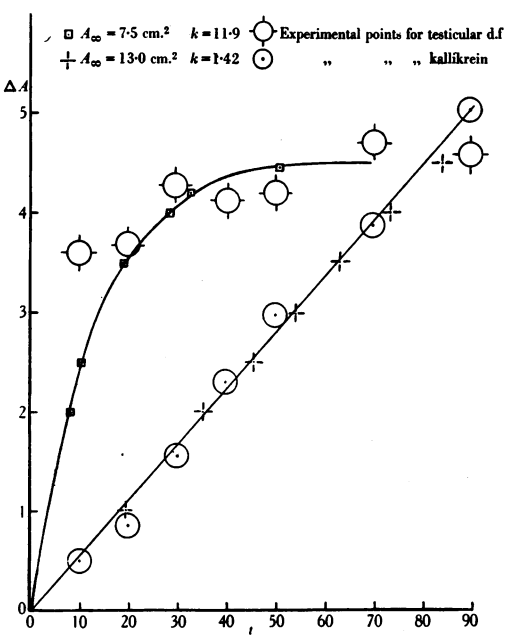

Fig. 6.

continuous lines values calculated from the equation using the magnitudes k for curve (i) 11·9 cm./min., k for curve (ii) 1·42 cm./min. and the ratio of

$$\frac{\rho \text{ curve (i)}}{\rho \text{ curve (ii)}} = \frac{1{\cdot}8}{1}.$$

The expression developed here while accounting satisfactorily for the rate of spreading does not account for the small effect which change of concentration of the diffusing factor has upon the area attained after some hours. If the mechanism outlined here were to hold until equilibrium had been achieved we should expect the final area to increase linearly with the concentration of diffusing factor molecules.

This is not the case as is seen from Fig. 1. The following may be advanced as a possible explanation. As well as the rapid reaction occurring between the diffusing factor molecules and the non-permeable dermal layer (which reaction determines the velocity in the early stages of the process) there is also a much slower reaction which can occur between the diffusing factor molecules and "wall" which has already been made permeable.

The nature of such a reaction is not yet clear, but if it were an adsorption process then in the later stages of the diffusion process, when the number of molecules of the dermal layer which have been rendered permeable is very large, the adsorption would lead to a more rapid loss of diffusion factor molecules. Consequently the final area achieved would be related to the initial concentration by

$$x_0 = \rho d\,(A_\infty - A_0) + f\,(A_\infty - A_0),$$

where the first term gives the amount of diffusion factor which has been removed by making the wall permeable and the second term that which has been removed by reaction with the substrate after it has been made permeable.

If the slower process is an adsorption process it may obey a law of the type $f\,(A_\infty - A_0) = k_2 x_0^{1/n}\,(A_\infty - A_0)\,d$ where k_2 and n are the constants of the adsorption isotherm when the increase in area at infinite time is given by

$$(A_\infty - A_0) = \frac{x_0}{\rho d + k_2 x_0^{1/n}}.$$

Fig. 7 shows the type of curve resulting from this relationship.

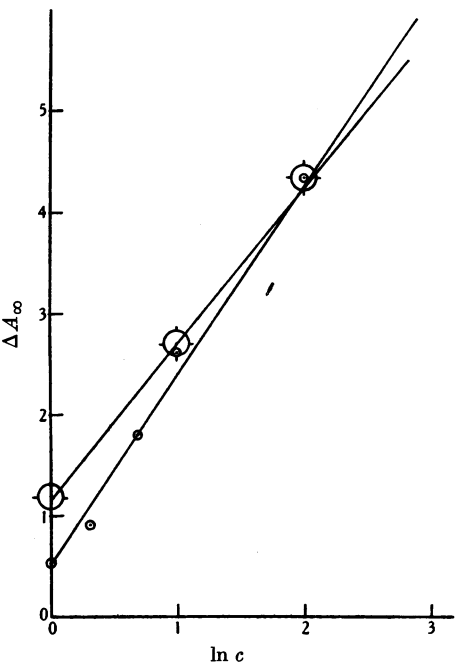

Fig. 7.

McClean *et al.* [1933; 1934] have studied the influence of testicular diffusing factor on the rate of absorption of diphtheria antitoxin when administered subcutaneously. They injected into guinea-pigs mixtures containing a fixed amount of antitoxin and varying quantities of diffusing factor, and observed that an increase in the concentration of the latter caused an increase in the number of units of antitoxin circulating in the blood of the animals. Since the testicular diffusing factor has no effect on the blood circulation [Madinaveitia, 1939] this increased passage of antitoxin into the circulation probably indicates that the diffusing factors increase the permeability of the subcutaneous layer just as they increase that of the dermis. This would cause the injected fluid to spread over a larger area and so increase the number

of capillaries coming in contact with it and capable of conveying the antitoxin. The type of dose-response curve obtained on plotting the experimental results of McClean *et al.* [1934] is similar to the dose-response curve obtained with coloured indicators in the skin of rabbits (Fig. 5).

SUMMARY

The mode of action of diffusing factors is discussed and a possible theoretical explanation advanced.

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