CCXXXV. STUDIES ON DIFFUSING FACTORS. ACTIVE PREPARATIONS FROM MAMMALIAN TESTICLE AND THEIR BIOLOGICAL ASSAY

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Aqueous extracts of mammalian testicles contain a factor which dramatically increases the permeability of the skin to injected fluids and particles [McClean, 1930; 1931; Hoffman and Duran-Reynals, 1931]. An intracutaneous injection of saline, serum or an extract of any organ other than testicle produces a well-marked bleb, the margins of which remain quite distinct for 20–30 min. A similar injection of testicular extract, on the contrary, immediately diffuses into the dermis so that after 30–60 sec. it is difficult to identify the site of injection.

It can be shown that this diffusing factor is active in very low concentrations in the following way. Ascending dilutions of the extract are mixed with a constant amount of indian ink or a solution of diphtheria toxin, and the mixture is injected intracutaneously into the shaved back of a rabbit. Control injections of indian ink or toxin mixed with saline are also made. It is then observed that the area over which the particles of indian ink spread, or the size of the cutaneous lesions due to diphtheria toxin, is increased by high dilutions of testicular extract.

The factor responsible for the effect is associated with the germinal epithelium of the testicle, and is also found in extracts of spermatozoa [McClean, 1931]. Subsequent work has revealed that factors with similar diffusing properties can be obtained from the most diverse sources; e.g. from filtrates of invasive strains of staphylococcus and streptococcus [Duran-Reynals, 1933], from organisms of the gas-gangrene group and virulent pneumococci [McClean, 1936], from extracts of malignant tissues [Boyland & McClean, 1935], from snake and spider venoms [Duran-Reynals, 1936] and from leech extracts [Claude, 1937]. It appears therefore that these diffusing factors may have considerable physiological and pathological importance, and their chemical isolation is desirable.

A method of purification of the diffusing factor from bull's testicles has been described by Morgan & McClean [1932]. They estimated the activities of different fractions by determining the highest dilution which would produce an increase of at least 20% in the area of the cutaneous lesion produced by a standard dose of diphtheria toxin. This method is not sufficiently reliable for quantitative assay of diffusing factors for the following reasons: the skins of the experimental animals vary in their susceptibility to the toxin and to the diffusing factor; no standard diffusing preparation is used against which samples of unknown potency can be compared in each animal; the time of evolution and the type of the toxin lesion varies in different animals, and therefore the area of the lesions is not always clearly circumscribed and cannot always be determined at the same interval after injection; it is quite impossible to distinguish between successive tenfold dilutions of a given solution, and it is doubtful whether hundredfold dilutions can be consistently differentiated.

For further purification of the diffusing factor a more accurate method of titration is required. The use of indian ink as an indicator for the quantitative estimation of purified samples has been criticised by Favilli & McClean [1934]. They found that particles of indian ink adsorb the partially purified diffusing factor and that the amount adsorbed varies in different preparations. Furthermore, when indian ink is used it usually remains accumulated at the site of injection, and only a small amount spreads round it forming a halo of faintly stained tissue. The boundaries of this halo are not sharp enough to allow accurate measurements. The ideal indicator would be a soluble colouring matter which is not absorbed by the skin tissues, which spreads uniformly but not excessively, and is not removed too quickly. Such properties are possessed in large measure by haemoglobin. During the first few hours following an intracutaneous injection of a solution of a foreign haemoglobin the deeply pigmented spot at the site of injection is surrounded by a halo of less pigmented tissue. The outer boundaries of this halo are sharp and can be accurately measured. After 2–3 hr. the haemoglobin is evenly distributed over the whole area, which is approximately elliptical in shape. The stained areas produced by intracutaneous injection into different rabbits of a fixed amount of haemoglobin solution containing a given concentration of diffusing factor are relatively constant in size. Thus if the same titration is repeated in several animals the average of the results will not be influenced by extreme values obtained in a particular rabbit.

The haemoglobin does not disappear from the skin for at least 30 hr. The rate of increase of the coloured areas during this time can be determined. It has been found that the area coloured by haemoglobin increases rapidly during the first hour, the rate of increase being proportional to the concentration of diffusing factor. After 2–3 hr. it becomes independent of concentration (Fig. 1). The area of a coloured patch produced by a certain concentration of diffusing factor is always larger than that corresponding to an injection of a hundredfold dilution. The results are reproducible if similar injections are made in different places on the back of the same rabbit. If the results obtained in three rabbits are averaged it is possible to distinguish between tenfold dilutions. The experimental error is too large to allow closer titrations (Fig. 2).

The following standard procedure was followed throughout this work.

To compare the activities of two preparations of diffusing factor a series of tenfold dilutions of each is prepared. An isotonic solution of haemoglobin is used as a diluent. Intracutaneous injections of each concentration of one preparation are made in one side of the shaved back of each of three rabbits. In the other flank injections of the corresponding dilutions of the second preparation are made. Dilutions containing the same concentration should give coloured patches of similar area and moreover their rates of spread should be the same (Fig. 3).

For testing solutions of unknown activity, comparison is made with a standard stable preparation of diffusing factor, stored in the form of a water-soluble powder. The diffusing activity of this standard has been determined by establishing the smallest concentration at which it produces a definite increase in the rate of diffusion of haemoglobin and in the area of tissue over which it spreads. Since the susceptibility of the skin of individual rabbits to the diffusing factor may vary somewhat, determinations were made on five animals and the mean of the results was taken as a measure of the diffusing potency of this preparation (Fig. 4).

A considerable concentration of the diffusing factor present in aqueous extracts of bull testicles has been achieved by Morgan & McClean [1932]. They

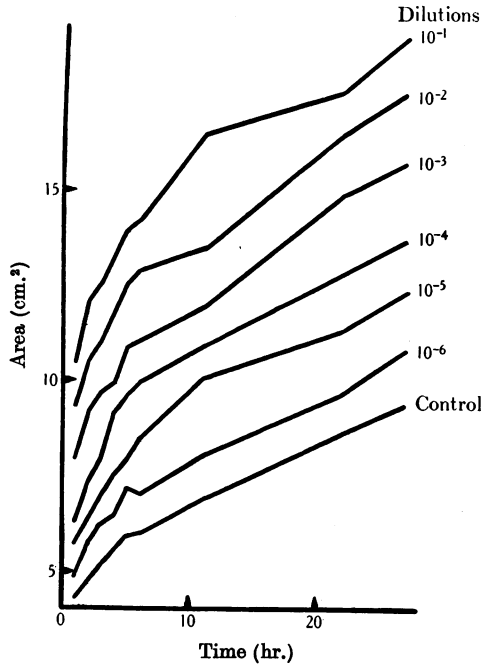

Fig. 1.

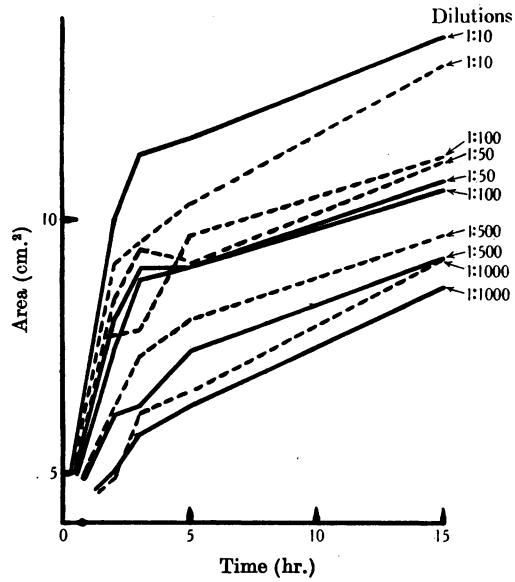

Fig. 2. —— Right flank. --- Left flank.

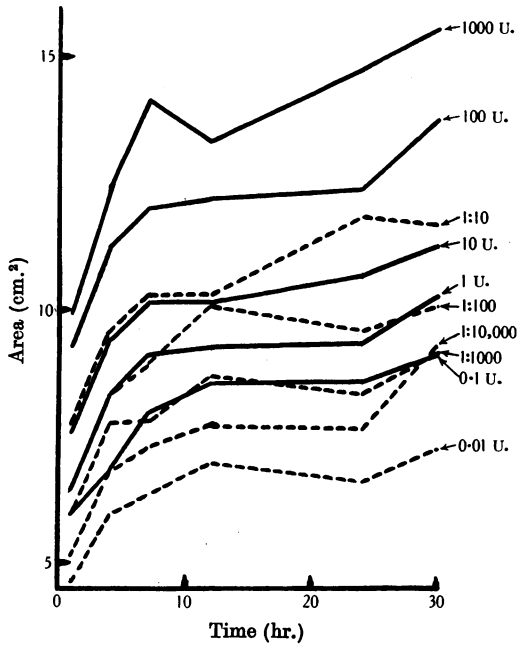

Fig. 3. —— Standard. --- Unknown.

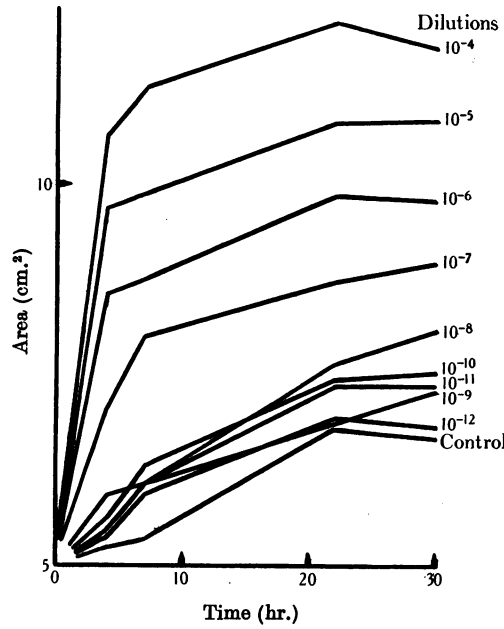

Fig. 4.

found that most of the protein could be removed from such extracts by precipitation with neutral lead acetate. From the filtrate the diffusing factor, together with $Pb(OH)_2$, was precipitated on making alkaline. The active material could also be precipitated with basic lead acetate. Claude & Duran-Reynals [1937] have described another method of concentration. This method involves treatment of crude testicular extract with acetone and fractionation of the redissolved acetone precipitate with $(NH_4)SO_4$. The active material precipitates with fully saturated but not with half saturated $(NH_4)_2SO_4$. Preparations obtained in this way obviously contain large amounts of protein, and, since Claude & Duran-Reynals did not employ a quantitative method of assaying biological activity, it is difficult to form any idea of the degree of purification attained by them.

Preparations containing relatively little protein have now been obtained by a method similar to that used by Morgan & McClean, save that the basic lead precipitate was decomposed with H_2S and the active material precipitated from the Pb-free solution with acetone. In agreement with the results of earlier workers, the diffusing factor was found to be non-dialysable (cellophane membranes) and insoluble in non-aqueous solvents with the exception of glacial acetic acid. Readily soluble in 50 % alcohol, it is insoluble at concentrations of alcohol greater than 70–75 %. The failure to dialyse must be regarded as a well established fact since as little as 0·01 μg. of active material per ml. can be detected biologically. This might indicate that the substance causing increase in dermal permeability has a high mol. wt.

The results of ultracentrifugal sedimentation, on the other hand, seem to indicate a relatively low mol. wt. Dr A. S. McFarlane (of this Institute), to whom my thanks are due, carried out an experiment in which a 2 % solution (pH = 6·2) of an active material (1000 units per ml.) was centrifuged. The density of this preparation was measured pyknometrically at 25° and found to be 1·40. Very little sedimentation was observed after 3 hr. in a field 300,000 times gravity. The degree of sedimentation observed was such that the mean mol. wt. could not be greater than 10,000. The boundary was not sharp.

Some of the effects of purified preparations of diffusing factor on compressed unimolecular films were studied by Dr J. Schulman (Cambridge) to whom I also wish to express my thanks. The surface potential of a gliadin monolayer was substantially changed on adding the preparation. The alteration observed was of nearly the same order as that produced by a similar concentration of saponin. Unlike saponin, however, the tested preparation had no effect on the surface potential of a cholesterol monolayer.

The stability of purified concentrates of diffusing factor in solution at different pH values has been studied. At pH 3 complete loss of activity occurred after 24 hr. at room temperature. No loss of activity occurred at pH 4, 5, 6, 8 or 9. At pH 11·5 noticeable destruction (about 90 %) was observed, while activity was completely destroyed after 24 hr. at pH < 3 or $> 11{\cdot}5$. Dilute solutions (0·1 %, pH 7) of the purest preparations available lost their activity rather easily on heating. At temperatures of 60° and over, more than 90 % of the activity was lost in 5 min. At 37° the solutions were much more stable, more than 90 % of the activity being retained after 24 hr.

These results appear to be at variance with those of Claude & Duran-Reynals [1937] and Aylward [1937] who found some of their preparations to be relatively stable. The fact that these workers did not use a quantitative assay method may explain their results, since large changes in the concentration of diffusing factor solutions cause relatively little change in the area over which haemoglobin spreads in experimental animals.

The purest preparations of diffusing factor so far obtained are obviously mixtures of different substances. The only pure compound so far isolated from them is *meso*-inositol. This substance crystallises on addition of alcohol to dilute aqueous solutions of purified preparations. It can also be isolated from them by vacuum sublimation. Neither inositol nor its phosphate, phytin, show any diffusing properties in biological tests.

Experimental

Isotonic solution of haemoglobin. A measured amount of defibrinated sheep's blood is centrifuged and the serum decanted off. After washing with isotonic saline the erythrocytes are lysed with distilled water and made up to the original volume of blood. To render this solution isotonic 0·1 vol. of 8·5 % NaCl is added. A solution prepared in this way is used throughout as a diluent for preparing dilutions of the material to be tested.

Injections. Young male rabbits with unpigmented skin are used throughout. Rabbits with coloured fur are preferable. The back and flanks of the animal are shaved with electric clippers.

The rabbits are gripped as for measuring the coloured areas (see below). A line is drawn along the backbone of the animal and each flank divided into a number of squares corresponding to the number of injections to be made. The length of back generally available is 15–20 cm. and the areas coloured by the haemoglobin have a diameter of 2–5 cm. Therefore only five injections can be made on each flank. In order to use most of the available space the injections on each flank are made alternately at 1 and 2 cm. from the backbone. Only one row of injections can be made on each side of the rabbit.

The material is injected intracutaneously and deeply coloured weals result. When high concentrations of diffusing factor are injected the weals flatten almost immediately and the haemoglobin spreads rapidly over a large area of skin. 0·3 ml. is injected into each site; smaller amounts give areas difficult to measure accurately, and when larger volumes are injected, the injected fluid is apt to leak back through the point of injection.

Measurement of the coloured areas. The rabbits are gripped by the ears and the upper part of the hind legs and held on the knees of a seated assistant. Unnecessary strain of the animal's body is to be avoided. The longest (D) and shortest (d) axes of every coloured area are measured ($\pm$ 1 mm.) with calipers. The area (S) is calculated assuming that the coloured patches are regular ellipses $\left(S=\pi\frac{D.d}{4}\right)$. Variations due to the position in which the rabbits are held can be partially avoided by releasing the rabbit and repeating the reading after a short time. The average of three consecutive readings is taken as the area of the stain.

Less than 5 min. are required to measure the areas on each rabbit. During the first hour following injection the coloured areas grow too rapidly to allow three successive accurate readings to be taken but during the later measurements the area does not alter appreciably in the time required.

Influence of the concentration of diffusing factor on the rate of spread of haemoglobin through the dermal tissues. From an aqueous solution (10 mg. per ml.) of a dry preparation of diffusing factor a series of tenfold dilutions was prepared, the diluent being in each case an isotonic solution of haemoglobin. Dilutions ranging from 10^{-1} to 10^{-6} were injected into the flanks of three rabbits. Two controls of haemoglobin alone were also injected. The average rate of spread of the different coloured areas is shown in Fig. 1. The area of the lesions is consistently proportional to the concentration of diffusing factor.

Reproducibility of the values. Into one flank of each of three rabbits a series of fivefold dilutions (in isotonic haemoglobin) of a solution containing the active material was injected. The same dilutions were injected into the opposite flank of the same rabbits. The rate of spread of the pigmented areas is shown in Fig. 2. The lines representing consecutive fivefold dilutions are not constantly arranged according to their concentration. When three rabbits are used the experimental error does not allow one to distinguish between fivefold dilutions. The limit of accuracy of this method appears to be tenfold dilutions.

The haemoglobin disappears from the skin in about 3 days. Identical injections were repeated after an interval of 4 days in the same areas of the animals which had been used in the previous experiment. The area and rate of spread of the coloured areas were much smaller and less regular than after the first injections. It is therefore inadvisable to use the same rabbits twice.

Activity of the standard preparation. A dry preparation obtained by a method similar to that described by Morgan & McClean [1932] is used as a standard. Serial tenfold dilutions of a solution (1 mg./ml.) of this preparation were made. Nine consecutive dilutions and a control of haemoglobin solution alone were injected into the flanks of a group of five rabbits. The lowest dilution contained 10^{-4} g./ml. and the highest 10^{-12} g./ml.

An average of the results obtained in the five animals was plotted graphically (Fig. 3). It will be seen that no definite diffusion was shown by solutions containing less than 10^{-8} g./ml. 0·01 μg. is the minimal diffusing dose of the standard preparation. 1 μg. of this powder is considered as a unit, and the activity of any preparation expressed in units per mg. or ml. Thus the standard preparation has one thousand units per mg.

Titration of a solution of unknown activity. Injections of a series of five successive tenfold dilutions of a solution of the standard preparation were made in one flank of each of a group of three rabbits. The strongest solution contained one thousand units per ml. (1 mg./ml.). Tenfold dilutions of the solution of unknown activity were injected into the other flank of the rabbits. The results are shown in Fig. 4. Dilutions of 10^{-1}, and 10^{-2} and 10^{-3} of the unknown solution produced coloured areas of similar size and rate of spread to those produced by the standard containing 10, 1 and 0·1 units per ml. Thus the diffusing activity of the solution under test was equivalent to 100 units per ml.

Concentration of the diffusing factor. The starting material used was either the testicles of recently killed bulls or a desiccated bull's testicle powder supplied by the Instituto Biologico Argentino, Buenos Aires.

Twenty-four fresh bulls' testicles (10 kg.), freed from the adjacent membranes, were minced in a meat mincer and the resulting pulp extracted with water (10 l.). Alternatively the dry testicle powder (1·5 kg.) was directly extracted with water (10 l.). After standing in the cold room (+5°) for 24 hr. the water was decanted off and the residue re-extracted three times more in a similar way.

A saturated solution of neutral lead acetate (1 l.) was added to the combined crude extract (40 l.) and the inactive precipitate removed. To the clear filtrate more neutral lead acetate (500 ml.) was added and then the solution made alkaline (*p*H 8·9) with ammonia. The active precipitate was collected. It was suspended in water (1 l.) and acidified (*p*H 4) with acetic acid. After removing the insoluble residue the Pb was precipitated by H_2S. When precipitation was complete the mixture was filtered and the filtrate concentrated *in vacuo* (bath temperature 37–40°) to half its original volume (500 ml.). Addition of acetone (4 l.) precipitates the active material quantitatively. The precipitate was extracted with water (50 ml.) and reprecipitated with acetone (500 ml.), the active

constituents now separating as a thick oil. This was almost completely soluble in water (50 ml.) and after removing a small amount of insoluble material the active constituents were precipitated as a white amorphous powder (4 g.) by addition of alcohol (250 ml.).

On account of the inaccuracy of the biological assay, even with the improvements described in this paper, it is difficult to obtain a precise idea of the concentration reached. The starting material (1500 g. of dry testicle powder) had some 10 units per mg. and the purified powder had 1000.

Purified preparations of diffusing factor are very readily soluble in water and slightly hygroscopic. They give a positive biuret reaction and a precipitate with trichloroacetic acid. The average percentage composition of the concentrate is C, 37·5%; H, 6·5%; N, 6·6%; O (by difference) 58·4%; P, 2% and 9% ash. They are completely soluble in 50% alcohol and partially in 70%, in the latter case only traces of diffusing activity going into solution. Soluble in glacial acetic acid, they are insoluble in all other non-aqueous solvents tried.

By addition of alcohol to a dilute solution of purified diffusing factor and keeping the solution for 3 months at room temperature an inactive crystalline material is obtained. The same inactive substance could be isolated from active preparations by sublimation *in vacuo* (200–220°/0·5 mm.). Recrystallised by adding an excess of alcohol to a concentrated aqueous solution or from hot 50% acetic acid it had M.P. 222–225°. (Found: C, 39·7; H, 6·8; $C_6H_{12}O_6$ requires C, 40·0; H, 6·7.) The substance showed all recorded properties of *meso*inositol and a mixed M.P. with an authentic specimen of this substance gave no depression.

Effect of the p*H on the activity of solutions of purified preparations of diffusing factor.* A solution (2 mg./ml.) of a purified preparation of diffusing factor (1000 units per mg.) was distributed in a series of tubes and each sample diluted with an equal volume of a 0·2 *M* buffer solution of the required *p*H. After 16 hr. at room temperature a small amount of each sample (2 ml.) was buffered with a 0·15 *M* phosphate buffer (*p*H 7·2, 8 ml.) and the resulting solution diluted ten times with isotonic solution of haemoglobin.

Into one flank of each of three rabbits the solutions which had been in acid medium were injected. A further three rabbits were used for the solutions which had been in an alkaline medium. The other flanks of the rabbits were injected with dilutions of the standard preparation having 100, 10, 1 and 0·1 units per ml. Control injections of haemoglobin alone were also made.

The rates of spread of haemoglobin injected with the solutions of diffusing factor which had been kept at *p*H 2, 3 and 14 were almost identical with that of the control injection of haemoglobin alone. The rate of spread of the one kept at *p*H 11·5 was similar to the one of the standard having 1 unit per ml. At *p*H 4, 5, 6, 8, 9 and 10 no destruction of activity was noticed, the haemoglobin spreading at the same rate as the solution of the standard preparation having 10 units per ml.

Effect of heat on the activity of solutions of purified preparations of diffusing factor. Two samples of a solution (1 mg./ml.) of a purified preparation (1000 units per mg.) of diffusing factor were heated at 60° and 80° respectively. Samples were taken out at 5, 15, 30 and 100 min. After cooling them as quickly as possible they were kept about 20 hr. in the cold room (+5°). Each sample was diluted ten times with an isotonic solution of haemoglobin and injected into one flank of two groups of three rabbits, each group corresponding to one temperature. The other flank of each rabbit was injected with dilutions of the standard preparation.

The rate of spread produced by the sample heated 5 min. at 80° indicated an activity corresponding to 0·1 unit per ml., whereas the unheated injected solution corresponded to 100 units per ml. Although 99·9 % of the activity was lost on heating the increase in the rate of spread of the area coloured by haemoglobin produced by the heated solution was nearly half of the increase produced by the unheated one which was a thousand times more active. 99 % of the activity of the sample heated at 60° was lost in 5 min. and about 99·9 % in 15 min.

Another sample of a similar solution (1 mg./ml.) of the same preparation (1000 units per mg.) of diffusing factor was kept in an incubator (37°) for one day. The activity of this heated solution was found to be nearly the same as that of the standard preparation (1000 units per mg.).

Summary

1. A new procedure is described for the biological assay of preparations of diffusing factor from mammalian testicle. This method, in which haemoglobin is used as an indicator, enables successive tenfold dilutions of diffusing factor to be differentiated with certainty.

2. An improved method for concentrating the diffusing factor is described, and preparations have been obtained, 0·01 μg. of which caused marked increase in tissue permeability.

3. From highly active preparations *meso*inositol has been isolated; it has no diffusing activity when tested biologically.

4. The activity of concentrates of diffusing factor is rapidly destroyed at temperatures above 37° and at *p*H below 4 or above 10.

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