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Chemical Nature of the Reynals Spreading Factor from Mammalian Testicle.

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The properties of the spreading factor found in aqueous extracts of mammalian testicle have been recently reviewed by McClean[1] and by Duran-Reynals.[2] This factor causes an increased permeability of the dermis to inert particles such as dyes and India ink, also to bacterial toxins, bacteria and viruses. How this effect is produced is not clear. It has been attributed to an alteration of the connective tissue barrier and some changes have been found in the histological appearances of the collagen fibers. It does not appear to be caused by an increased flow of lymph; the property can be demonstrated in excised skin as long as 48 hours after death. Aside from mammalian testis, the "spreading factor" has been demonstrated in other tissues, such as brain, liver and kidney, but in relatively small amounts. It is present in considerable quantity in certain bacteria, in certain tumors, in certain snake venoms and insect venoms; the factor is closely associated with the venom but can be dissociated from it.

Attempts to ascertain the chemical nature of the spreading factor have been made along two lines. A great variety of pure chemical substances have been examined for spreading properties, in most cases with negative results. Some spreading action is found in the case of glycerine, triacetin, with certain lecithins and with some commercial peptones. The most encouraging observation along this line has been with azoproteins. Various chemical studies have been made upon the testicular extracts which have thrown some light upon the chemical nature of active agent, and have suggested that it is more closely related to proteins than to lipoids or carbohydrates: The active principle is precipitated by ammonium sulphate, by basic lead acetate, by alcohol and ether, and by acetone. It is soluble in water and in dilute acids. It is relatively thermostable, resisting 100°C. for several minutes in the moist state and higher temperatures when dry. It passes through a

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1 McClean, D., *Biol. Rev.*, 1933, **6**, 345.

2 Duran-Reynals, F., *Ann. de l'Inst. Pasteur*, 1936, **57**, 597.

Berkefeld filter, but is apparently non-dialysable. It is inactivated by trypsin; however, autolysis of certain tissues like brain in which it is present in small quantity, causes an increase in the concentration. Although purified preparations have been made which are active in a dilution of 1:1,000,000, none of these products have been protein-free. The experiments here reported were undertaken to confirm and extend knowledge of the chemical properties of the spreading factor.

For the purification of the extract the method suggested by Claude and Duran-Reynals was used. Minced testicles were extracted with n/10 acetic acid, the filtered extract being precipitated by acetone.† The acetone precipitate was dried, dissolved in water, filtered and reprecipitated by acetone, the process being repeated 10 times in all. The various acetone precipitates were studied independently, being tested for potency by observing their effect on the spread of a suspension of India ink in physiological saline or m/50 phosphate buffer (pH 7.1) injected intradermally into the flank of rabbits. Control injections were made in all instances.

The first acetone precipitate (yield 21.0 gm. per kilo of testicle) was a light brown powder, not completely soluble in water. The second acetone precipitate (yield 2.6 gm. per kilo of testicle) was colorless and almost completely water soluble. A slight amount of suspended material was present which could be removed by a Seitz No. 3 filter. Subsequent precipitates were similar in character and showed very little diminution in yield and no perceptible gain in potency. Most of the work described below was done with the second and third acetone precipitates.

Preliminary tests were done which confirmed data in the literature as to the properties of the spreading factor. In powdered form it was stable at room temperature, but solutions deteriorated rapidly unless kept in the icebox. On heating the solutions to 100°C. for 5 minutes, potency was completely lost by the first acetone precipitation (apparently because of adsorption by the large amount of protein coagulated by this procedure) but the more purified products when subjected to this treatment yielded negligible coagulation and retained their potency.

The extracts were soluble in concentrated KOH and in glacial acetic acid in the cold. With strong mineral acids the material went into solution only on warming with the development of colors (violet with HCl, pink with H_2SO_4; deep yellow with HNO_3).

† This first step of the process was carried out for us by the research laboratory of E. R. Squibb & Sons, through the courtesy of Dr. John F. Anderson.

When dissolved in distilled water the acetone precipitates yielded solutions with a pH between 6.2 and 6.9; the solutions were well buffered.

Qualitative tests for carbohydrates, lipoids and proteins were carried out. A slight alpha naphthol test was given by all the active fractions, and after hydrolysis with HCl the solution showed a slight reducing action. Thus small amounts of carbohydrate may be present—free or in combination. The precipitates were insoluble in fat solvents, but in order to exclude the possibility of lipoid complexes being present, material was treated with alcoholic KOH, acidified and then extracted with ether. A negligible amount of material (inactive) was found in the ethereal extract. The Xantho-proteic, Millon's, Hopkins-Cole glyoxylic acid reaction and diazo reactions were all positive; a weak biuret test was obtained. The active material was precipitated from solution by protein precipitants: ammonium sulphate, mercuric chloride, trichloracetic acid and lead acetate. Thus, in confirmation of previous work, it appears that the active material is at least intimately associated with protein.

Elementary analyses of the first, second and third acetone precipitates were made‡ in the hope of demonstrating a shift in composition coincident with purification:

	C	H	N	S	P	Ash
First Acetone Precipitate	44.50	6.42	12.31	0.92	2.24	11.70
Second " "	37.99	5.87	11.63	0.83	3.81	20.00
Third " "	38.33	5.86	11.47	0.98	3.89	19.73

When calculated on an ash-free basis the analyses show somewhat lower C and N percentages than are obtained for typical proteins, but they fail to show any consistent shift in composition with purification. The most striking change is the increased percentage of ash. All preparations gave a test for inorganic phosphorus. Only a slight direct test for sulphur was obtained with sodium nitroprusside, but after fusion with sodium and extraction with water, a definite positive test was obtained indicating that most of the sulphur was in organic combination.

An attempt was made to see whether the active material could be inactivated by X-rays or ultraviolet rays. A 1% solution of second acetone precipitate (filtered through Seitz No. 3 filter) was exposed for one hour, being given 9000 roentgen units. At the end of this

‡ For the C, H, N and S analyses we are indebted to Prof. Hans T. Clarke of the Dept. of Biological Chemistry, Columbia University, in whose laboratory they were performed.

time it was unchanged in appearance and showed no loss in activity. A similar solution in a shallow pan was exposed for ½ hour to a mercury quartz arc at a distance of 10 cm. At the end of this time a precipitate had formed. Both precipitate and solution were completely inert.

Although it has been reported that the Reynals factor is not dialyzable, it appears that dialysis experiments were not carried out at varying hydrogen ion concentrations. We prepared 1% solutions which were placed in parlodion bags and dialyzed for 48 hours against citrate buffer solutions of pH 3, 5 and 8. The external medium was changed every 12 hours. In no case did active material appear to pass through the membrane. In a further experiment dialysis was carried out against a continuous flow of water. The material inside the membrane retained its activity. It thus appears that the Reynals factor either has a high molecular weight or is intimately associated with material of high molecular weight.

An attempt was made to study the behavior of the spreading factor in an electric field. A 5% solution of the second acetone precipitate was placed in a U-tube and a current of 5 milliamperes was passed through for 24 hours. At the cathode a brown protein-containing precipitate formed which settled to the bottom of the tube; it was found to be almost completely inactive. The solution at the cathode was tested after neutralization and was found to have lost the greater part of its activity. The solution at the anode, however, after neutralization was found to possess all the activity of the original solution. It thus appeared that the active material either was negatively charged and migrated to the anode or else was inactivated at the cathode by the precipitation of protein which took place there.

We then attempted to purify the active material by electrodialysis. A 5% aqueous solution of the third acetone precipitate was placed in the central portion of a Bradfield 3-chamber electrodialysis cell. Distilled water was placed in the 2 outer compartments containing the electrodes. The chambers were separated by parlodion membranes. A current of 10 milliamperes was passed through for 24 hours. No obvious change occurred in the outer chambers, but in the center a white precipitate settled out. The supernatant fluid remained a pale yellow color. At the end of the experiment the precipitate and the fluid in the 3 compartments were tested for activity. The precipitate and the fluid in the outer compartment containing the electrodes were inactive, whereas the clear fluid in the central compartment retained all the original activity. The precipitate appeared to be protein in nature, and its formation is

apparently due to a decrease in solubility caused by the passage of electrolytes into the lateral chambers containing the electrodes.

Further purification of the liquid in the central compartment was attempted by putting fresh distilled water in the outer chambers and passing the current through again. A further precipitate then settled out which on testing proved to be inactive. The supernatant fluid had retained its full activity. On evaporation of the supernatant fluid a solid was obtained which represented only 1/10 of the original material and contained only a very small percent of ash. Elementary analyses of this material are in progress.

Conclusion. Electrodialysis appears to be a very promising method for the purification of the Reynals spreading factor.

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Inhibition of Estrous Cycle in the Rodent with Post-partum Urine and Commercial Prolactin.

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In attempting to ascertain the effect of urine from lactating women upon mammary glands of rats and mice, it was observed that there was an inhibition of estrus in these animals. Animals with normally recurring estrus were used, and these had daily vaginal smears for at least 3 consecutive cycles as a means of control. Injections of post-partum urine were then started. It was found that at least one, usually 2, and occasionally 3 cycles were suppressed, after which the animal resumed its normal estrous rhythm in spite of continuation or increase in the amount of injected urine. Controls, injected with the urine of normal human males and females had no alteration of the rhythm. The rodent's ovaries during this period of induced diestrus contained active corpora lutea which were similar to the ovaries of lactating animals. If injections were discontinued during this period of induced diestrus, a vaginal smear indicative of estrus was obtained within 48 hours. These findings suggested that a sub-

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