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The Inhibitory Effect of the Lipoid Fraction of Tissue Extracts upon Cellular Proliferation

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by

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I. INTRODUCTION

A. Acknowldegements

To Dr. F. A. McJunkin, whose invaluable criticism as advisor and kindly consideration as a friend have stimulated me in this line of endeavor, I express my sincere appreciation.

For his cheerful cooperation and indispensable technical assistance in the preparation of tissues for microscopic examination, I gratefully thank Mr. L. M. Herdegen.

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It is through the kind permission of Mr. C. D. Hartman that the cytological effects of the extracts upon the liver are available for this report.

B. Purpose of the Problem

The work to be presented is an outgrowth of the experimental studies on cellular proliferation which have been in progress in the Department of Pathology during the

past several years. Last year a growth inhibitor was demonstrated in extracts of the kidney. It was the original purpose of the investigation to find a more efficient method of extraction of this inhibitory principle. Initial experiments indicated a close association of the inhibitor with the lipoid fraction of tissue extracts. Systematic attempts were then made to isolate the lipoids in maximum quantities for further study.

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V. SUMMARY

II. LITERATURE

The existence of substances in the blood and tissues that exert a retarding effect upon cellular proliferation is an established experimental fact. Carrel (1) noted that the growth of connective tissue cultivated outside the body was more abundant in the plasma of young chickens than in that of older animals. In 1921 Carrel and Ebeling (2) determined that the action of age on serum is characterized, not by the presence of an accelerating factor for the multiplication of fibroblasts, but by the increase of an inhibiting factor. Later, in 1923, the same authors (3) separated the serum into a growth activating and a growth inhibiting fraction by precipitation with carbon dioxide. The inhibiting substance was found to be heat resistant at 65° C. and remained in the serum with the albumin fraction.

The abnormal growth associated with tumor formation has also been shown to be influenced by tissue extracts. Sittenfield and Johnson (4) demonstrated a loss of the tumor producing properties of an active filtrate of the Rous chicken sarcoma after it had been mixed with the blood. The active agent could be recovered, however, by buffering the mixture to a pH 4.0 and then extracting

the resultant precipitate at a pH of 8.0. This substance that neutralized the tumor principle was subsuquently found by Sittenfield, Johnson and Jobling (5) to be confined to the globulin fraction of the serum.

The evidence cited suggests the existence of an inhibitory mechanism functioning within the body during the normal process of cellular proliferation, and several specific substances are known to exert in vivo an anti-growth effect. By thyroid feeding, Gray and Loeb (6) produced a retardation of the mitotic rate in the thyroid glands of guinea pigs. McJunkin and Roberts (7) found that excessive insulin inhibited proliferation in the pancreatic islet cells of young rats. Parenteral administration of adrenaline has a similar depressant effect on karyokinesis in the suprarenal medulla as was shown by McJunkin, Singer and Rall (8). In 1932 McJunkin, Tweedy and Breuhaus (9) demonstrated an inhibition of the mitotic activity of the parathyroid gland by the injection of activated or inactivated parathormone. The authors concur in assigning to the endocrine glands studied a self regulatory action of the respective secretion, an excess of circulating normone causing a decrease in secretion and mitotic activity. Last year McJunkin and Hartman (10) reported the presence of a growth inhibitor

in various extracts of the kidney, an organ not known to have an internal secretion.

With the exception of the hormones, very little is known of the nature of the various anti-growth principles which have been demonstrated by these investigators. The assignation of an inhibitory role to the lipoid constituents of tissues is not without experimental support. Goldfarb (11) and Robertson (12) noted a marked retardation in the development of fertilized sea urchin eggs when lecithin was added to the culture medium. Lecithin was found to exert a depressant effect upon the division rate of paramecia by Browder (13). Local injections of an aqueous emulsion of lecithin greatly retards the growth of carcinoma in rats, according to Robertson and Burnett (14). Decrease in body weight, indicative of growth inhibition, was demonstrated in mice fed with lecithin by Robertson (15). A similar retardation in suckling mice, following the feeding of lecithin to the mother, was recorded by Robertson and Cutler (16).

III. MATERIALS AND METHODS

Part 1. Preparation of Extracts

Most of the extracts were made from rat kidneys. Old animals were etherized and bled by severing the great vessels of the neck. After rapid, aseptic removal, the kidneys were stripped free of their fibrous capsules, together with the peripelvic fat and connective tissue. They were then ground in a mortar to the consistency of a smooth paste. In the preparation of dessicates, the macerated material was thinly spread on a glass plate and along the sides of the mortar, and then dried by playing an electric fan over it for one to two hours. The resultant hard scale was easily removed by a razor blade, transferred back to the original mortar and ground thoroughly. The powdered material was stored in bottles over phosphorous pentoxide. On several occasions beef kidney was used. It was obtained at the slaughter house, immediately iced and transported to the laboratory with the least possible delay. The defatted kidney was reduced to a workable state by passing it through a meat grinder several times. When other tissues, such as heart, were used, practically the same procedure was followed.

High grade, commercially pure reagents were employed

as extraction media, the time, temperature and concentration varying with the individual experiments. Recovery of the extracted material was obtained by evaporating the extracting agents, using a high speed fan at room temperature to hasten the process. Inasmuch as most of the reagents used were of a highly volatile nature, this procedure was usually accomplished in a few hours.

Part 2. Method of Bioassay

A measured portion of the extracted material was suspended in sterile physiological saline solution and tested. In all experiments a single intraperitoneal injection was made and the animal killed twenty-four hours later. Suckling albino rats varying in age from thirteen to forty-three days were used in the test. The best results were obtained with rats eighteen to twenty-one days old, as they were sufficiently resistant to infection and had a high mitotic rate. At least two, and sometimes three, litter mates were saved for controls. This was necessitated by the fact that the mitotic rate is neither constant with age nor body weight. After injection, the litter was returned to the mother until the time of autopsy, with no change in environ-

mental or dietary conditions.

The animals were killed and the right kidney immediately removed and fixed in formalin for no less than twelve hours. A three millimeter block, cut transversely midway between the poles, was embedded in paraffin for sectioning. Ribbons of sections, cut at eight microns thickness from the center of this block, were mounted and stained with hematoxylin and eosin. The mitoses present in the renal tubules in two or three sections were counted. If a great discrepancy in the counts occurred, several other sections were examined for a more correct mean. Recently the liver has been removed simultaneously with the kidney for fixation. A circular block eight millimeters in diameter was punched out of the larger left lobe by means of a sharp hollow tube, embedded in paraffin, sectioned, mounted and stained after the manner described. A microscopic enumeration of the mitoses present in the hepatic cells of one or two sections was made.

Part 3. Compilation of Results

It was early recognized that some system should be adopted for the tabulation of results in the light of dosage, strength and efficiency of extraction. The follow-

ing system was devised and found useful because of its simplicity. All doses were converted to the equivalent amount of fresh tissue represented by the extracted material. Knowing the fresh tissue equivalents of the substance injected, it was possible to compute the percentage reduction in the mitotic rate per gram of fresh tissue by comparing the mitotic counts of the experimental animals with the average number of mitoses observed in the controls. Since the figure obtained was based upon the effect manifested in several animals it served as a reliable indication of the inhibitory nature of the extracts, and had the additional advantage of affording a convenient method for graphic representation of the results obtained.

IV. RESULTS

McJunkin and Hartman (17) obtained the greatest retardation of the mitotic rate of the renal tubular epithelium with acid alcohol extracts of the kidney made alkaline to Congo red and clarified by acetone, a dose equivalent to three grams of fresh kidney substance being markedly inhibitory. With removal of the lipoid material and subsuquent purification of these extracts, a product was obtained which was quite potent in doses equivalent to thirty-six grams of fresh tissue. It was evident that there had been a considerable loss of the inhibitory substance or substances in the manipulations that followed clarification by acetone. With this fact in mind a systematic search was instituted to find, if possible, a more efficient method of extraction.

Because of the greater knowledge of kidney extracts and the obvious futility of extending the investigation to other tissues until a fairly satisfactory method of extraction had been discovered, the majority of the experiments conducted dealt with kidney tissue, either fresh or dessicated. In the recording of results, the factor system previously described was resorted to as frequently as possible because of its simplicity and uniformity. An

attempt has been made to arrange the experiments in convenient groups without disturbing the natural train of thought that has prevailed throughout the problem.

Part 1. The Inhibitory Effect of Simple Lipoid Extracts of the Kidney

The initial quest for a more efficient method of extraction assumed the nature of a rather exhaustive determination of the solubility of the inhibitory substance in various extraction media. Since the majority of the reagents employed fall into the classification of ordinary lipoid solvents, the appelation bestowed on this group of experiments seems justifiable. As the title indicates, the extracts tested were all of a more or less simple nature. The results of several of these extracts are plotted according to their factors in Table 1.

Extract 1. Fresh macerated kidney was mixed with two volumes of physiological saline solution and strained through muslin. The cloudy aqueous suspension was tested.

Extract 2. The chloroform soluble portion of the dried residue of a simple aqueous suspension similar to Extract 1.

Extract 3. The chloroform soluble portion of dried kidney which had been mixed with two volumes of an acid aqueous mixture of pH 4.6 for ninety-five hours at icebox temperature.

Extract 4. A 90% acetone extract prepared by covering fresh macerated kidney with ten volumes of acetone for twenty-five hours and then centrifuging.

Extract 5. The ether soluble portion of dessicated kidney that had been extracted with ether in a Soxhlet extractor for six hours.

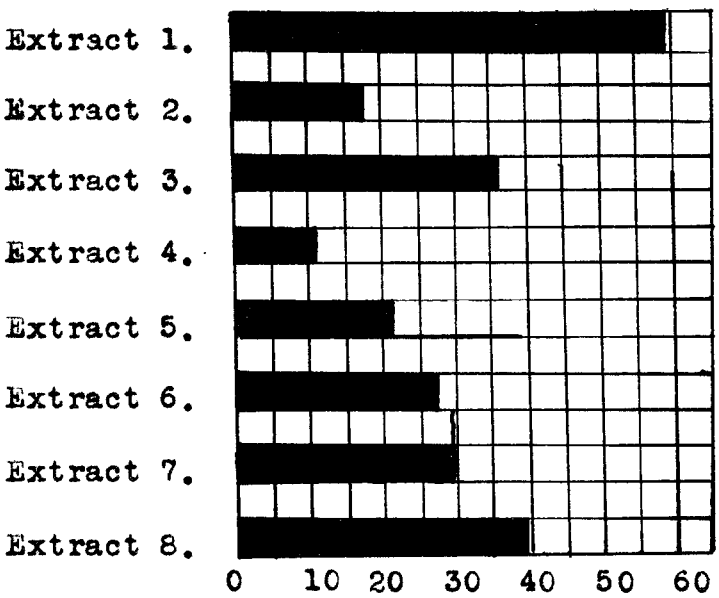
Extract 6. A 63.3% alcohol extract collected by centrifugation of a mixture of fresh kidney and two volumes of 95% alcohol which had been standing at room temperature for twenty-two hours.

Extract 7. The 86% alcohol filtrate obtained by covering fresh macerated kidney with ten volumes of 95% alcohol for three hours at room temperature.

Extract 8. A 95% alcohol extract derived from a mixture of dried kidney pulp and ten volumes of 95% alcohol which stood at room temperature for seventeen hours.

Table I

The Inhibitory Effect of Simple Lipoid Extracts of the Kidney



(Per cent reduction of the mitotic rate per gram of fresh tissue represented in units of five)

With the exception of the first simple aqueous suspension, all the succeeding extracts represented in Table I were clear, indicating that the extracted material was in solution. This encouraging fact coupled with the partial removal of the inhibitory substance by ether, chloroform and alcohol in high concentrations seemed to indicate that the inhibitor was of a lipoidal nature. The poor yield with extraction by acetone was also of favorable import because it tended to eliminate neutral fats

and cholesterol as inhibitory agents. Maclean (18) mentions the fact that lecithin and allied substances, although purported to insoluble in acetone, are removed to some extent by acetone solutions containing neutral fats. Perhaps the slight inhibition manifested by the acetone extract can be explained in this light.

Part 2. Attempts at Concentration of the Inhibitor

The success obtained by McJunkin and Hartman (17) with acid alcohol extracts, the increased yield in chloroform extracts after preliminary acidification and the significant results of high alcoholic concentrations, as Table I indicates, suggested a combination of acid hydrolysis and lipoid extraction. Several experiments were performed, three of which are depicted graphically in Table II.

Extract 9. Fresh kidney pulp was mixed with three volumes of a 0.2% solution of HCl in 60% alcohol and allowed to stand at room temperature for eighteen hours. Sufficient alcohol was then added to raise the alcoholic concentration to 86.6%. Separation of the clear extract was obtained by filtration.

Extract 10. The chloroform soluble portion of the dried residual kidney after Extract 9.

Extract 11. Practically the same procedure followed as in Extract 9, but combined with a second 95% alcohol extract.

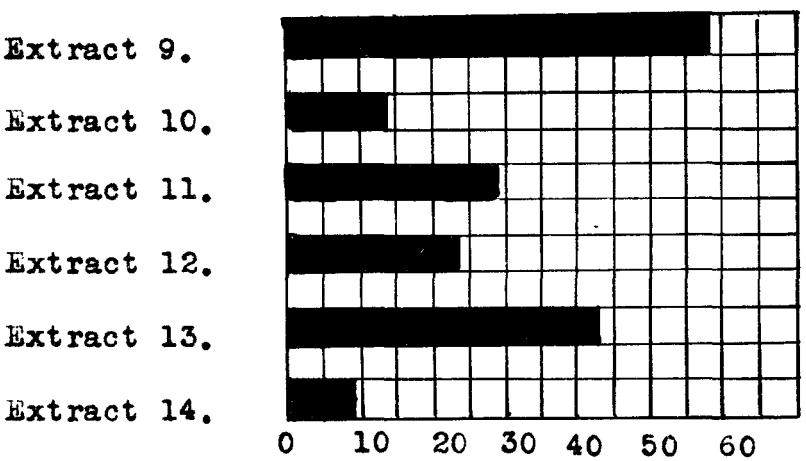
Extract 12. Same as Extract 11, using beef kidney.

Extract 13. The chloroform soluble portion of Extract 12.

Extract 14. The chloroform insoluble portion of Extract 12.

Table II

The Inhibitory Effect of Acid Alcohol Extracts of the Kidney



(Per cent reduction of the mitotic rate per gram of fresh tissue represented in units of five)

The results substantiated quite well the anticipations

of this method of combined extraction. The double acid alcohol extracts are as a whole more efficient than the single alcoholic extracts. Acidification apparently aids the process, probably by slightly hydrolyzing the protein particles and thereby allowing a more intimate contact with the extraction media. Of greater significance are the facts disclosed by Extracts 4, 5 and 6. The inhibitory effect of these double acid alcohol extracts is mainly due to their lipoid content, since removal of the lipoid material results in a substance that is relatively non-inhibitory, (Extract 14 Table II).

In order to rule out the possibility of any carry-over of split protein products that might have occurred in the acid alcohol extracts and to minimize nitrogenous contamination in general, experiments were designed along the lines of classical lipoid extraction as given by Maclean (18). Table III summarizes the results of two of these experiments.

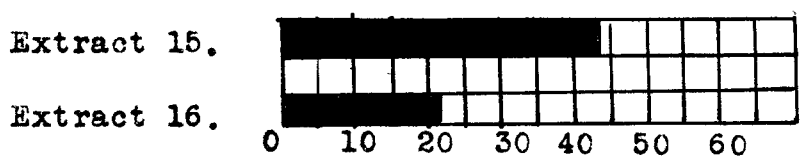
Extract 15. Fresh rat kidney pulp, dehydrated by acetone, was covered with ten volumes of chloroform for three hours at room temperature. The extract separated by filtration was combined with the filtrate obtained after the residual kidney tissue

had been mixed with ten volumes of 95% alcohol for seventeen hours.

Extract 16. Fresh beef kidney which had previously been extracted with acetone was covered with ten volumes of ether for forty-five hours at room temperature. The ether filtrate was added to a successive alcoholic extract collected by filtering a mixture of the kidney residue and ten volumes of 95% alcohol which had stood for ninety-six hours.

Table III

The Inhibitory Effect of Typical Lipoid Extracts of the Kidney



(Per cent reduction of the mitotic rate per gram of fresh tissue represented in units of five)

The apparent discrepancy between the respective efficiencies of Extracts 15 and 16 may be explained on the basis that large amounts of beef kidney were used in preparing Extract 16, thus enhancing the source of error in

computations. Furthermore, the kidney pulp after passage through the meat grinder contains much more coarse material than does the ground pulp used in extractions of small quantities of fresh tissue; hence the chances of intimate contact with the extraction media are diminished. When these factors are taken into consideration, it is evident that this type of extraction ranks higher in efficiency than any of the preceding. There is a close approach to the inhibition manifested by a simple aqueous extract, (Extract 1, Table I). Although this is very gratifying, the residue of the extracted kidney is still inhibitory, indicating that the present method of extraction is not complete. The difficulty of obtaining complete removal of the lipoid fraction of tissues is mentioned by both Wells (19) and Maclean (18). Wells goes so far as to state that only previous peptic digestion will release all of the lipoid constituents. At the present time a concentrated attack is being made on this phase of the problem in an endeavor to wrest more of the lipoid from the tissue.

Part 3. Specificity of the Inhibitor as Regards Occurrence and Action in Tissues other than the Kidney

In view of the difficulty encountered in attaining a method of extraction which would give a maximum yield of the inhibitor, little time could be devoted to this important consideration. Table IV illustrates the results obtained with identical extracts of the kidney and heart.

Extract 17. Dessicated kidney was covered with five volumes of chloroform for seven hours at room temperature. The extract was separated by filtration.

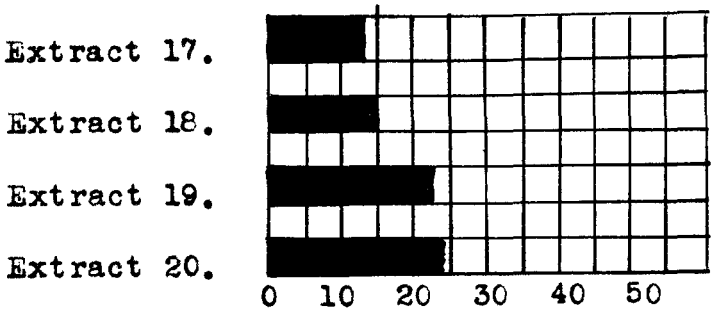
Extract 18. After removal of Extract 17, the residual kidney pulp was mixed with five volumes of 95% alcohol and allowed to stand for twenty-one hours at room temperature. The filtrate was saved.

Extract 19. Dessicated heart was treated in a manner similar to that used in preparing Extract 17.

Extract 20. An extract, analogous to Extract 18, was prepared from the residual heart tissue.

Table IV

The Inhibitory Effect of Lipoid Extracts of the Kidney and Heart



(Per cent reduction of the mitotic rate per gram of fresh tissue represented in units of five)

There is little doubt that the inhibitor is not of a specific nature at least in regard to occurrence, since heart extracts are just as effective, if not more so, in retarding the mitotic rate of the kidney. This finding is in accord with the unpublished results of McJunkin and Hartman who demonstrated the inhibitory effect of acid alcohol extracts of the liver, heart and mammary gland upon karyokinesis in the renal tubular epithelium.

Unfortunately, sufficient liver counts have not been made to warrant any really conclusive deductions, but there is a strong indication that this inhibitory substance exerts a similar retarding influence upon cellular proliferation in the hepatic cells. The effect of four

extracts upon the kidney and liver is shown in Table V.

Extract 21. Fresh kidney pulp was covered with three volumes of a solution of 0.2% HCl in 60% alcohol for sixteen hours at room temperature. Sufficient alcohol was then added to raise the alcoholic concentration to 86.6%. The clear filtrate was then combined with a second 95% alcohol extract of the residual kidney.

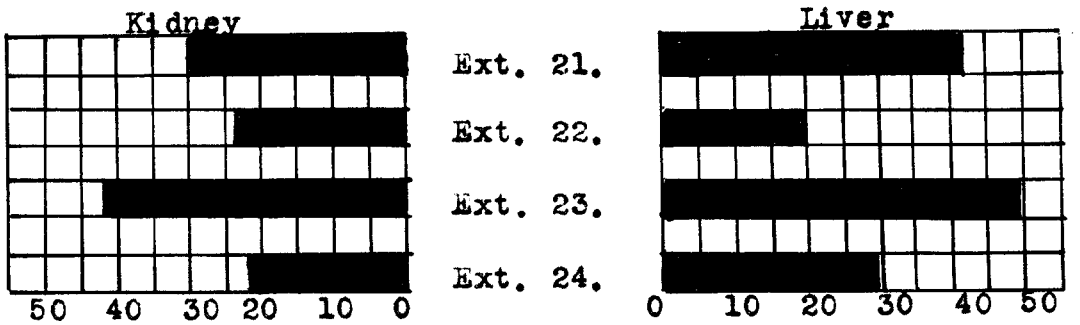
Extract 22. Essentially the same as Extract 21.

Extract 23. The chloroform soluble portion of Extract 22.

Extract 24. To fresh beef kidney which had previously been covered with ten volumes of acetone, were added ten volumes of ether and the mixture allowed to stand at room temperature for forty-five hours. The ether extract, separated by filtration, was combined with a second alcoholic filtrate obtained by covering the residual kidney with ten volumes of 95% alcohol for ninety-six hours.

Table V

The Inhibitory Effect of Lipoid Extracts upon the Kidney and Liver



(Per cent reduction of the mitotic rate per gram of fresh tissue represented in units of five.)

Part 4. General Properties of the Inhibitor

During the course of the investigation, several factors have been brought to light which may serve to identify the inhibitor with the lipids or some substance that cannot readily be separated from them by the use of solvents. The latter is likely of the nature of a nitrogenous contaminant such as the "carnithin" described by Maclean (18). Determinations of the nitrogen content of ether alcohol extracts disclose an average value of 3.12%. The average phosphorous content of the same extracts is 2.79%. The N:P ratio is, therefore, somewhat greater than in the purified phospholipids.

The inhibitor is resistant to heating at 65° C. for one hour. In this respect it behaves similarly to the growth inhibitor demonstrated to be present in the serum of animals by Carrel (3). Treatment of kidney pulp with several volumes of an aqueous 3% HCl solution at 70° or 100° C. for one hour does not destroy its inhibitory action. This relative stability towards heat is also suggestive of a lipid-like substance.

V. SUMMARY

In view of the experimental evidence presented, the following conclusions are made.

1. The lipoid fraction of tissue extracts contains a substance which exerts an inhibitory influence upon cellular proliferation.
2. The inhibitory substance is non-specific as regards occurrence and action.
3. The inhibitory substance is probably a lipid.

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