

114 (1046)

A method of studying the effect of serum upon tissues.By **S. FELDSTEIN, M.D.**

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In the investigation of the action of serum ferments on animal tissues chemical methods have been almost exclusively used. Within the last few years the dialysis method developed by Abderhalden has been extensively employed in experimental as well as clinical investigations. While the technique of this method is not as difficult as it is usually assumed to be, at every step of the procedure a not inconsiderable number of errors are likely to creep in. It requires very careful preparation of the substrate, absolutely uniform dialyzing tubes and great care in boiling the dialysate.

The most weighty objection to the dialysis method, however, lies in the fact that it gives only indirect evidence of the enzymatic action of the serum on the tissue. It is due to this fact that recently Bronfenbrenner and others have claimed that a positive Abderhalden test does not show the proteolytic action of the serum on the tissue substrate at all, but that during the incubation of the serum with tissue, the antiferment of the serum becomes adsorbed. The removal of the antiferment exposes the serum proteins to the action of the ferments in the serum and it is their cleavage products which pass into the dialysate and give the positive reaction.

Considerations of this nature led the author more than a year ago to seek for a method of demonstrating the ferment action of serum in the histologic examination of tissues. A priori it was to be expected that early evidence of the chemical changes resulting from ferment action might be shown by alteration in the microscopic appearance and staining reaction of the tissue.

Almost the first specimen examined, that of a boiled guinea-pig placenta exposed to the action of normal and pregnant guinea-pig serum, having fortunately shown a most remarkable difference, the author was encouraged to continue the investigation. Soon

it became evident that the histologic method was so delicate an indicator that unmistakable evidence of the action of normal serum on animal tissue was readily demonstrable. The activity of the serum varies greatly with the species of animal from which the serum is derived. Of those investigated the serum of the cat is apparently one of the most powerful and it is with the normal serum of this animal that the present investigation was chiefly concerned.

The serum was obtained from the jugular vein and immediately centrifuged; in this manner best avoiding the mechanical hemolysis which is likely to occur. At first, in addition to the boiled tissue, we attempted to use formalin and acetone fixed sections but found that these fixatives almost entirely inhibited the action of the serum.

In our more recent work we have used fresh and boiled tissue almost exclusively. The use of fresh tissue theoretically did not promise much, as, on the authority of Abderhalden, it has long been assumed that only in the boiled state do the tissue proteins undergo digestion. Moreover, the work of Longcope and others has shown that serum has a preservative action on tissue. This author, however, calls attention to the fact that in the most superficial layers of the block of tissue immersed in serum, the nuclei do not stain, and it is apparently these changes that we are concerned with in our present investigation. In the use of fresh tissue, the factor of autolysis is involved. But as Launoy has shown with the guinea-pig liver, and as we have been able to confirm, marked histologic evidence of autolysis does not appear within the first 24 hours, if the temperature is kept below 37.5 degrees C. Our sections were incubated from 18–21 hours, and in only a few instances did our controls show marked autolytic changes, when needless to say that particular experiment was rejected.

When boiled tissue was used as substrate, small blocks about 2 cm. in thickness were cut, washed in water, then thrown in physiological salt solution and boiled for fifteen minutes. Frozen sections, 10 microns in thickness, were made of the fresh and boiled organs.

The activity of the serum was determined by a series of dilutions, 1, 1/10, 1/20, etc., in physiological salt solution.

The sections (all of about the same size and thickness) were placed in small test tubes, each containing 1 c.c. of the serum dilution. 1 c.c. of physiological salt solution was the constant control. To each tube was added 1 c.c. of toluol and the whole series incubated at 37.5° C. for 18–21 hours.

The staining was done by means of Delafield's hematoxylen and eosin. All sections of a series were stained in an identical

Delafield's hematoxylen.....	1 min.
½ per cent. alcohol eosin.....	10 sec.
85 per cent. alcohol.....	30 sec.
Oil of origanum.....	Until clear.
Canada balsam mounting.	

manner. Fresh sections were treated by floating on a slide, drying on blotting paper over the paraffin stove, then staining. Boiled sections were transferred in the moist state to the various solutions, as otherwise they are likely to float off the slide. We have also employed the various fat stains and the Altmann granule stain but have found the simple hematoxylen-eosin adequate.

The most striking changes are found in the nuclei. When exposed to the action of cat serum up to a dilution of 1 : 30, no nuclei are to be seen in the boiled sections. On fresh tissue, contrary to what might have been expected, this action was even more marked, as no nuclei were visible at a dilution of 1 : 120 of the serum. The nuclei either had disappeared entirely or had failed to take the stain. In addition the tissue appeared cloudy, parboiled, and not infrequently cleavage lines ran through the whole specimen. At times, as in the guinea-pig placenta to be shown on the screen, the organ assumed a reticular structure, as in specimens treated by the Spalteholz or Mall's digestion method. At other times, the whole specimen appeared homogeneous, the tissue becoming unrecognizable. Fibrous tissue as the trabeculæ of the spleen and adventitia of vessels stand out distinctly, but the nuclei seem to have disappeared. The appearances were very similar to those observed in autolysis of organs.

When we had established that the titre of activity of cat serum remains constant, *i. e.*, no nuclei in the boiled sections on exposure to a dilution of 1 : 30 or 1 : 40 we injected a cat whose serum was previously tested, with 1 gm. of fresh cat liver freed from blood,

intraperitoneally three times, and tested its serum against cat liver. The action of the serum after the series of injections had increased so that the specimens exposed to a dilution of 1 : 60 resembled histologically the ones previously exposed to 1 : 30, etc. The action on the spleen was not nearly as marked.

The serum of a rabbit, injected with Beebe's nucleoproteids derived from dog thyroid, at a dilution of 1 : 5 had a much more striking action on the fresh dog thyroid than on the dog spleen.

As to the exact interpretation of these changes: They cannot be attributed to bacterial action as toluol was always added in abundant amounts and we have found that not infrequently bacteria failed to grow on culture media when they were kept in the same incubator owing to the evaporation of the toluol. Moreover, in a series examined at intervals of ten minutes, we have found that at the end of an hour, the changes in the histologic appearance of the sections were quite marked; a period of time too short for bacterial action to have taken place. We have also been able to show that while inactivation at 55° C. for one hour failed to destroy this activity of the serum, exposure to 65° C. for one half hour led to complete loss of activity.

From the appearance of the specimens, it is my belief that a number of ferments might be concerned in the production of these striking changes, not only proteases but also peptases and perhaps esterases and nucleases. Sodium fluoride in 0.3 per cent. solution, which is a specific inhibitor of esterase action seemed to weaken the activity of the serum. With normal serum extracted by chloroform according to the method advised recently by Jobling, we have not obtained uniform results. When the serum after filtration remained turbid, as was often the case, no action on the tissue was demonstrable. This activity of the serum is apparently not identical with that of trypsin. With the latter, there is marked fragmentation of the sections at a time when nuclei are still visible. While the present investigation was in progress, there appeared a brief preliminary communication in the *Muenchener medicinische Wochenschrift*, No. 37, 1914, by H. Rollett, describing a method by which bits of boiled placenta were exposed to serum, then examined histologically. Very meager details are given.

Dr. W. L. Rost has assisted me in most of the experiments of the present investigation.

115 (1047)

The effect of sensitization on pneumococcus lesions of the lobar type in rabbits.

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For the purpose of ascertaining to what extent conditions of hypersusceptibility determine the character of pneumococcus lesions of the lung, series of experiments were made with a moderately virulent strain, and with an extremely virulent strain in its virulent state and after artificial attenuation. Rabbits were inoculated intravenously for purposes of sensitization with .1-15 c.c. pneumococcus filtrates or dead cells and then after two weeks injected tracheally with 1. c.c. live cultures. The animals surviving 48 hours were killed. Microscopic sections were made of all lungs.

In these experiments with attempted active sensitization none of the animals developed symptoms resembling anaphylactic shock nor was the lung involvement definitely increased in any series of previously treated rabbits. Tracheal injection of the moderately virulent organisms, however, caused marked lesions in both sensitized and unsensitized control rabbits.

In experiments with attempted passive sensitization, mixtures of 1 c.c. virulent or attenuated live cultures and .1 c.c. or .5 c.c. sera from normal or immunized rabbits when injected tracheally failed to incite uniformly extensive lesions in any series of animals though the proportion developing diffuse involvement was greater than in the previous experiments with active sensitization. Sudden paroxysms, similar to those of fatal anaphylactic shock were observed about twenty-four hours after tracheal injection in practically all the animals of two or three experiments. But these paroxysms were not associated with extensive lesions of the lung because in a number of the rabbits no characteristic exudative pneumonias were found, although the lungs were almost invariably deeply congested.