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Effect of Ischaemic Necrosis and Autolysis *in vitro* on the Soluble Proteins of Rat Kidneys

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The structural changes undergone by the cells in the course of coagulation necrosis have been investigated in detail by many authors (Arnheim, 1890; Heidenhain, 1907; Schmaus & Albrecht, 1895; Schottlander, 1888, 1891). Although many papers have been published on the biochemical changes occurring during the necrotic process, an explanation, on a biochemical basis, of the events taking place in this pathological condition is still lacking. In particular, the data so far available on the fate of the protein components of the cells in this condition are essentially analogous as they deal mainly with the autolysis *in vitro* under aseptic conditions. These investigations deal particularly with the behaviour of various enzyme systems and with the substances appearing during the hydrolytic cleavage of the proteins (see the extensive reviews of Bradley, 1922, 1938; Pozzi, 1935). The disappearance of the cellular proteins, in the course of necrosis, was thus referred to digestion of the protein components due to proteolytic enzymes still active in the dead cells (Bradley, 1922, 1938).

No investigations on the biochemical changes of the soluble proteins, during the course of necrosis, have been carried out so far by direct analytical procedures. In this paper the soluble proteins of normal kidneys and of kidneys in the course of ischaemic necrosis and autolysis *in vitro* have been studied, mainly by paper electrophoresis.

EXPERIMENTAL

Male albino rats of the Italico strain, weighing 150–180 g., bred in this Department and fed on a standard diet (Randoin & Causeret, 1947), were used.

Ligation of the renal vessels. Ligation of the left renal artery and vein was carried out, under light ether anaesthesia, with sterile silk thread. To ensure partial drainage of the blood trapped in the vessels of the organ, the renal vein was severed distally to the ligature.

Transplantation of the kidneys to the peritoneal cavity. Animals were killed by exsanguination under light ether anaesthesia and the body was perfused through the ascending aorta with about 200 ml. of sterilized ice-cold 0.9% NaCl soln. The kidneys were removed, weighed and transferred under aseptic conditions to the peritoneal cavities of other rats.

Aseptic autolysis in vitro. Organs perfused as described above were put in sterilized glass-stoppered test tubes containing some drops of chloroform. Direct contact of the antiseptic was avoided by suspending the organs on small glass rods. Autolysis was carried out in an oven at 37°.

Extraction of the soluble proteins. The weighed organs were dispersed in 1 vol. of water, adjusted to pH 7.8, with a Potter-Elvehjem glass homogenizer kept in crushed ice. The dispersion was centrifuged for 1 hr. at 25 000 g in a refrigerated angle centrifuge (Espirouette Phywe, Phywe A.-G., Göttingen) and the supernatant fluid again centrifuged for 1 hr. with the same centrifugal force. The supernatant fluid was stored in the cold room at 2° and used for the electrophoretic analysis.

Determination of the total and soluble protein contents of the tissue dispersions. Proteins from the whole homogenate and supernatant fluid were precipitated with 5% (w/v) trichloroacetic acid; digestion was performed in the presence of potassium persulphate; NH_3 was distilled in the Markham (1942) apparatus and collected in 0.0143 N- H_2SO_4 for back titration with 0.0143 N-NaOH, Tashiro's indicator (King, 1951) being used.

Paper electrophoresis. This was carried out in an horizontal-strip-type of apparatus with a boric acid- Na_2SO_4 -NaOH buffer [0.154 M, pH 8.6, I 0.12 (P. Adjutantis, personal communication).]

Strips of paper (Whatman no. 1; 12 cm. $\times$ 30 cm.) were allowed to equilibrate in the apparatus and then the fluids were applied as a narrow band near to the cathode end. Separation was carried out with a constant potential of 135–150 v and a current, at the beginning of the experiment, of about 4 mA for a single strip. The strips, dried in an oven at 110° for 30 min., were stained with Amido-black 10B according to Grassman, Hannig & Knedel (1951). The relative percentages of the separated components were

determined by cutting out the appropriate portions of the stained strip, eluting the dye with 10 ml. of phenol soln. (4%, w/v) in 0.02 M-NaOH and reading the optical density in a photoelectric colorimeter with a 610 m μ filter.

RESULTS

Soluble proteins of normal kidneys

The supernatant fluid obtained from normal perfused organs was slightly opalescent, yellow-brown in colour, with an average protein content of 2.90 g./100 ml. The supernatant from normal unperfused organs showed instead a red-brown colour, due to the presence of haemoglobin; its protein content was 3.9 ± 0.4 g./100 ml.

Fig. 1a shows the electrophoretic patterns of the soluble proteins of normal, perfused kidneys. The six separated components are distinguished with the numbers from 1 to 6 in order of their increasing mobility. The protein components nos. 4 and 5, in some experiments including the one illustrated, did not separate well because of their similar mobilities. Moreover, proteins electrophoretically not homogeneous, with high mobilities, migrate ahead of fraction no. 6 (Fig. 1a). They represent $24.3 \pm 1.0\%$ of the total soluble proteins; with greater potential differences, this material can be resolved into two fairly distinct bands. Thus the total number of the protein components in the supernatant fluid from perfused normal kidneys is at least eight. Fig. 1b shows the electrophoretic pattern of the supernatant fluid from unperfused normal kidneys. A comparison of Figs. 1a and 1b shows that the supernatant fluid from unperfused organs differed from that of the perfused only by the presence of an additional component migrating ahead of the other fractions. Moreover, the separation of the components nos. 4 and 5 is still more difficult because of the presence of additional material migrating with approximately their mobility. Other experiments showed that the

component with high mobility, present in the supernatant fluid from unperfused kidneys, shows the same mobility as rat-serum albumin, whereas haemoglobin and β -globulins of blood serum, after electrophoresis, are located approximately in the interval between the components nos. 4 and 5. It was therefore concluded that the differences between the fluids obtained from perfused and unperfused organs were due to the presence of blood in the latter.

In Table 1 the relative percentages of the various components from perfused and unperfused normal kidneys are reported. In view of the poor separation of the components nos. 4 and 5, these fractions were determined together; the same procedure was followed for the components nos. 7 and 8.

Soluble proteins of the kidney, after ligation of the blood vessels

Proteins were extracted after 4, 8, 24, 48, 72, 96 and 118 hr. of experimentally induced ischaemia. No modifications were observed in the electrophoretic patterns after 4 hr. Later, the material migrating between fraction no. 6 and the albumin band was decreased, whereas the other components were less clearly resolved. After 24 hr. an increase

Table 1. *Relative percentage distribution of the soluble-protein components of perfused and unperfused rat kidneys, separated by paper electrophoresis*

Results are means from nine individual experiments $\pm$ S.E.M.

Fraction no.	Perfused kidneys	Unperfused kidneys
1	8.9 ± 0.5	7.7 ± 0.3
2	11.1 ± 0.4	10.6 ± 0.4
3	12.4 ± 0.8	10.7 ± 0.5
4+5	21.0 ± 0.4	21.3 ± 1.1
6	22.1 ± 0.8	21.1 ± 1.2
7+8	24.3 ± 1.0	20.4 ± 1.0
Albumin	—	7.1 ± 0.5

Fig. 1. Electrophoretic patterns of the soluble proteins of kidneys of rats: a, Normal, perfused kidneys; b, normal, unperfused kidneys; c, kidneys after 24 hr. in the peritoneal cavities of other rats; d, kidneys after 48 hr. in the peritoneal cavities of other rats; e, kidneys after 72 hr. in the peritoneal cavities of other rats.

of the albumin band had begun, which was more evident at 48 hr.; also at this time a component with the mobility of serum β -globulin was evident. After 72 hr. the electrophoretic pattern of the soluble proteins was similar to that of the blood serum, whereas all the components of the cell proteins, previously observed, had disappeared. This might indicate that proteins from interstitial fluid had penetrated the necrotic tissue. For a direct demonstration of this hypothesis, the following experiments were performed.

Soluble proteins of perfused kidneys which had been transferred to the peritoneal cavity of other rats

The modifications of the weight and of the protein contents of kidneys transferred to the peritoneal cavity are reported in Fig. 2. From this it is apparent that the weights of kidneys kept in the peritoneal cavity of living animals underwent a slight increase in the first 24 hr. and an important decrease afterwards. Fig. 2 shows also that the soluble proteins underwent a rapid decrease in the

first 24 hr.; later, this decrease was slower. Fig. 1c-e represent the electrophoretic patterns of the soluble proteins of organs kept respectively for 24, 48 and 72 hr. in the peritoneal cavity. The patterns were similar to those already observed after ligation of the blood vessels *in situ*. At 24 hr. (Fig. 1c) an albumin fraction was first visible, and the protein components migrating between the latter and component no. 6 were decreased; the other components, with the exception of fraction no. 6, were not clearly distinguished. At 48 hr. (Fig. 1d), a further increase in the albumin component was evident. Moreover, the presence of a fraction with a mobility similar to that of β -globulins was noted. At 72 hr. (Fig. 1e), all the original kidney proteins were absent and the electrophoretic pattern was similar to that of the blood serum of the same animals.

Soluble proteins of the kidney during autolysis in vitro

These were studied after 4, 8, 24, 48, 72 and 96 hr. of autolysis. Fig. 3 shows the total protein contents of the tissue dispersion and of the corresponding supernatant fluid. It is apparent that during the first 8 hr. a rapid decrease in the soluble protein levels took place, after which the decrease continued at a slower rate. From a comparison of Figs. 2 and 3 some differences in the behaviour of the soluble proteins of kidneys during the autolysis *in vitro*, and of those of kidneys kept in the peritoneal cavity are apparent. The levels of soluble protein from organs kept in the peritoneal cavity were always higher than those of organs kept *in*

Fig. 2. Modifications of the weight and of the protein content of the kidneys after transplanting into the peritoneal cavities of other rats. ○, Wet wt. of the organs, expressed as percentage of the original weight; △, total protein contents of the tissue dispersion (1:2); □, protein contents of the supernatant fluid, after centrifuging (see text). Each experimental point corresponds to the mean of from three to seven individual results. The vertical lines represent twice the standard error of the means.

Fig. 3. Effect of the autolysis *in vitro* on the protein content of the kidneys. ▲, Total protein content of the tissue dispersion (1:2); ●, protein content of the supernatant fluid after centrifuging (see text). Each experimental point corresponds to the mean of from three to seven individual results. The vertical lines represent twice the standard error of the means.

vitro at 37°. Moreover, in necrosis *in vivo*, the rapid decrease in the soluble proteins was evident only during the first 4 hr., whereas in the autolysis *in vitro* it was observed for the first 8 hr. The electrophoretic patterns observed during the course of autolysis showed only a progressive decrease of the original protein components.

DISCUSSION

The results here presented show that the soluble proteins extracted from perfused normal renal tissue of rats are separated by paper electrophoresis into eight components. In the supernatant fluid from unperfused organs an additional component, similar to serum albumin, was detected. Moreover, in the latter case the presence of haemoglobin and of material similar in mobility to serum β -globulin was demonstrated. Results for the soluble proteins of necrotic kidneys demonstrate that during necrosis a progressive decrease of these proteins occurred, accompanied by the progressive appearance in the necrotic organs of other soluble proteins. These proteins, on the basis of their number, relative percentage distribution and mobility, appear identical with the serum proteins. Possibly, proteins analogous to those of the blood serum pass in this condition from the interstitial fluid into the necrotic tissue. This interpretation is substantially in agreement with Verzani's (1936) findings, confirmed in the present investigation, that the necrotic tissue undergoes an increase in weight. However, this weight increase takes place in the first hours and, according to Verzani (1936), is probably due to imbibition of interstitial fluid. The passage of the foreign proteins into the necrotic organ is detectable only after 24 hr. and is coincident with the reduction in the weight of the organs.

The present data permit a better understanding of the phenomenon of ischaemic necrosis as compared with aseptic autolysis *in vitro*. In this connexion some published results point to biochemical differences between these two conditions. Thus whereas autolysing tissues (Bradley, 1922, 1938; Sevringhaus, Koehler & Bradley, 1923) and those in the first stage of ischaemia (Rous & Drury, 1929) show a pH more acid than normal, Borger, Peters & Kurz (1933), Leuthardt & Koller (1934), Baldassi (1935) and Verzani (1936) have demonstrated that the ischaemic necrotic tissue shows a neutral or alkaline pH. Verzani (1936) adds that necrotic rabbit kidneys are increasingly acid during the first hour after the beginning of ischaemia, whereas later they became neutral and eventually alkaline. It has also been demonstrated that the activities of some enzymes are increased in the course of autolysis *in vitro*, whereas they are

reduced in the necrotic tissues (Borger, Bajerle, Mayer & Peters, 1935; Pozzi, 1935; Verzani, 1936). The present investigation shows that, during the course of necrosis, blood proteins pass into the tissue. In view of the inhibitory action of serum proteins on tissue proteolytic enzymes (Bradley, 1922) and of the possibility that, together with the electrophoretically demonstrated proteins, serum enzymes might also penetrate into the necrotic tissue, the phenomenon described here could have a notable influence on the biochemical events taking place in this tissue.

SUMMARY

1. The soluble proteins extracted from perfused kidneys of normal rats have been separated by paper electrophoresis into eight components. It was demonstrated that the supernatant fluid obtained from unperfused, normal kidneys contains, in addition to a haemoglobin band, a component similar in mobility to serum albumin, as well as a faint band probably identical with serum β -globulin.

2. Perfused kidneys transferred to the peritoneal cavities of other rats increase in weight during the first 24 hr., and then decrease in weight rapidly. The total soluble proteins of the kidneys decrease rapidly during the first 4 hr. of ischaemia and remain practically constant afterwards. In autolysis *in vitro*, the rapid decrease in soluble proteins lasts for the first 8 hr. and is more marked than in the previous case; later, a slower decrease is observed.

3. Paper electrophoresis of the soluble proteins extracted from kidneys left *in situ* after ligation of the blood vessels, and from kidneys transferred to the peritoneal cavities of other rats, shows a progressive disappearance of the original tissue proteins, accompanied by the appearance of foreign proteins similar to those of the blood serum of the same animals; such proteins were not observed in autolysis *in vitro*.

4. The interpretation of these findings is discussed.

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The Biochemistry of Organotin Compounds

THE CONVERSION OF TETRAETHYLtin INTO TRIETHYLtin IN MAMMALS

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In their study on the toxicity of organotin compounds Stoner, Barnes & Duff (1955) noted that when tetraethyltin was given to rabbits the symptoms after an initial latent period closely resembled those of triethyltin poisoning. They suggested that tetraethyltin was converted into triethyltin. A similar description of the symptoms of tetraethyltin poisoning in rabbits, particularly muscular weakness and lowered body temperature, has been made by Lecoq (1954) and a whole series of these tetra-alkyltin compounds has been studied by Meynier (1956). Previous work (Cremer, 1957) has shown that there is a close correlation between the amount of triethyltin given to rats, the concentration attained in the brain and the degree of inhibition of the metabolism *in vitro* of brain slices prepared from treated rats. An analytical procedure for determining triethyltin in biological media was also described. By similar techniques to those used in that study it has now been possible to show that tetraethyltin is converted into triethyltin by rats and rabbits. Studies on the conversion mechanism *in vitro* have been made and the liver has been found to be the organ most active in converting tetraethyltin into triethyltin.

MATERIALS AND METHODS

The animals used were albino rats of 200–230 g. body wt., maintained on M.R.C. diet no. 41 B (Bruce & Parkes, 1956), and cross-bred rabbits fed on M.R.C. diet no. 18 (Bruce, 1947).

Brain slices were prepared and used as previously described (Cremer, 1957).

Preparation and fractionation of liver homogenates. Fractionation of a 10% (w/v) liver suspension in ice-cold 0.15 M-NaCl–0.015 M-MgSO₄–0.008 M-nicotinamide (Davison,

1955) was essentially by the method of Schneider (1948). Liver was homogenized in a Potter–Elvehjem type of homogenizer with a Perspex pestle, as described by Aldridge (1957). Cells and nuclei were removed by centrifuging at 600 g for 10 min., mitochondria by a further 20 min. at 5000 g and microsomes by a further 120 min. at 20 000 g. Each sediment was resuspended without washing to half the original volume of the whole homogenate. For routine experiments the supernatant from a 10% (w/v) liver homogenate in NaCl–MgSO₄–nicotinamide medium, centrifuged for 10 min. at 5000 g, was used.

Protein was estimated by the biuret method of Robinson & Hogden (1940) as modified by Aldridge (1957), with bovine-serum albumin as the standard.

Purification of tetraethyltin. Tetraethyltin was obtained from Dr G. J. M. van der Kerk. Triethyltin impurity in the sample was removed by several extractions of a solution of tetraethyltin in CHCl₃ with N-H₂SO₄ in subdued light, followed by washing with water, borate–ethylenediamine–tetraacetic acid (EDTA) buffer (Na₂B₄O₇·10H₂O, 19 g.; boric acid, 12 g.; EDTA, 4 g.; in 1 l. of water. pH 8.4) (Cremer, 1957), conductivity water and evaporating the CHCl₃ *in vacuo*. The purified sample was stored in the dark at 0°. A stock ethanolic solution of tetraethyltin (40 mg./ml.) was prepared and stored in the dark at 0°. This solution was used for intravenous injection. For addition to Warburg flasks small samples of the ethanolic solution were pipetted rapidly into larger volumes of the saline media and shaken vigorously, the suspension being prepared just before use.

Estimation of triethyltin. Triethyltin was estimated in biological material by the dithizone method previously described (Cremer, 1957). The percentage recoveries for the various tissues given in that work have been used to calculate the triethyltin/g. of tissue wet wt. Purified tetraethyltin does not form a complex with dithizone (Aldridge & Cremer, 1957). For experiments *in vitro* triethyltin was estimated in the total flask contents and expressed as µg. found/g. wet wt. for liver slices or µg./100 mg. of protein in cell-fractionation experiments.