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A BIOCHEMICAL APPROACH TO THE STUDY OF REJECTION OF CANINE RENAL HOMOTRANSPLANTS

III. Histochemical Studies of Transplanted Kidneys

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In a previous paper (Tyler, Williams, Kountz, Kapros and Dempster, 1964), we reported some enzyme changes occurring in transplanted kidneys. It was shown in quantitative studies that a depression in the levels of some enzymes occurs immediately after transplantation. Such losses of enzyme activity may be recoverable, but homotransplants show further decreases commencing some time before oliguria. In relatively long surviving homotransplanted kidneys enzyme activity may begin to fall after day 4 or 5, while in kidneys surviving for shorter periods, enzyme activities become depressed within the first 4 days. Hydrolases show a different behaviour to respiratory enzymes, since, while respiratory enzymes reach a minimum at oliguria-anuria, hydrolases may rise after an early fall to reach 140 per cent of the normal level in kidneys which survive for periods of more than a week.

The factors involved in these changes in enzyme activity have been further studied using enzyme histochemical techniques. The results of these further investigations are presented in this paper.

MATERIALS AND METHODS

Surgery.—Kidneys were transplanted and later removed during the functioning phase or at oliguria as previously reported. (Tyler *et al.*, 1964).

Preparation of tissues.—Several small blocks containing cortex and cortico-medullary region, were quickly frozen in liquid oxygen, sealed in heavy gauge polythene bags, and stored at −40° until required. The blocks were allowed to warm to −15° before cutting at 6–8 μ in a Bright cryostat, at −15° to −20°. Sections were allowed to dry for 3–5 min. at

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room temperature after collecting on a cover glass. After drying, the sections were incubated immediately or stored at −20° for a short period. The material examined consisted of 6 normal kidneys, 5 autotransplants, 4 functioning and 5 oliguric homotransplants. The oliguric homotransplants included those which rejected between the 5th and the 17th day.

Enzyme demonstration.—Fresh (unfixed sections) were used to demonstrate the following enzymes :—(1) Cytochrome oxidase by the method of Burstone (1960), and Person, Burstone and Fine (1962). The incubation time was 60 min. at 25°. (2) Succinic dehydrogenase by the " MTT " of Pearse (1960) using an incubation time of 30–40 min. at 37°. (3) Malic dehydrogenase by the " MTT " + " Nitro BT " methods of Pearse (1960) using an incubation time of 60 min. at 37°. (4) DPNH diaphorase by the " MTT " method of Pearse (1960) using an incubation time of 20–30 min. at 25°. In all cases where the methods of Pearse (1960) were used Polyvinylpyrrolidone was omitted from the medium.

Sections fixed in cold absolute acetone (0–4°) for 1 hr. were used for the demonstration of the following enzymes : (1) Acid phosphatase by the method Burstone (1958) using an incubation time of 30–40 min. at 37°. (2) β-glucuronidase using the method of Seligman, Tsou, Turenberg and Cohen, (1954) using an incubation time of 6 hr. at room temperature. (3) N-acetyl-β-glucosaminidase was demonstrated using Naphthol-AS-LC-N-acetyl-β-glucosaminide as substrate. The medium consisted of the following reagents : 4 mg. of naphthol-AS-LC-N-β-glucosaminide dissolved in 1–2 drops of dimethylformamide ; 12·5 ml. of 0·05 M phosphate-citrate buffer pH 4·5 ; 12·5 ml. of water ; 25 mg. of Garnet GBC. After filtration the medium was added to the sections and they were incubated for 20–30 min. at 37°.

All sections were mounted in glycerine jelly after they had been finally fixed in 10 per cent formalin to stop the reaction.

Assessment of Enzyme Activities.—Comparison of normal and transplanted kidneys was always performed by staining transplants simultaneously in the same jar with at least two normal kidneys.

Additional stains.—Oil Red-O was used to examine a number of normals and autotransplants.

Material for methyl-green-pyronine stains was obtained as serial sections to those used for enzyme demonstrations, the cryostat sections being first fixed in 10 per cent formalin-saline. Paraffin sections of formalin fixed material were used for the preparation of haematoxylin and eosin, toluidine blue and periodic acid-Schiff stains.

RESULTS

The distribution of enzymes in the normal dog kidney

Acid phosphatase.—In all regions of the cortex the activity in the proximal convoluted tubule is confined to small spherical droplets or granules (Fig. 1), distributed fairly evenly throughout the cell. Straighter segments of the proximal tubules in the medullary rays bore droplets less numerous in number and nearer to the lumen than in the proximal convoluted tubule. In the distal tubule very few tiny droplets were seen. The glomeruli had a very faint reaction.

β-glucosaminidase.—The localisation of the reaction product was not easy to assess because of the crystalline nature of the azo dye formed. A number of diazonium salts were tried, but they seemed to inhibit the enzyme. Garnet GBC gave a satisfactory result but this was not ideal. The product is fairly granular and is heaviest in the proximal convoluted tubule throughout the cortex. In the proximal tubule of the medullary rays, the reaction is less, and is very faint in the cortical distal tubule. Faint glomerular staining was occasionally seen. The distribution agrees fairly closely with that seen with other substrates (Pugh and Walker, 1961).

β-glucuronidase.—The reaction obtained with the post coupling method was unsatisfactory when applied to dog kidney, there being little or no deposition of reaction product after six hours incubation. This appears to be a problem of

TABLE—*The Histochemical Demonstration of some Enzymes in Normal Dog Kidney*

Enzyme	Blood Vessels	Glom.	Aglomerular cortex		Glomerular cortex		Labyrinth cortex		Medullary ray		
			PCT	DT	PCT	DT	PCT	DT	PT	LH	CD
Acid phosphatase	−	±	++++	+	++++	+	++++	+	+++	±	±
β-glucosaminidase	−	±	+++	+	+++	+	++	+	++	−	+
Succinic dehydrogenase	±	−	+++	++++	+++	++++	+++	+++	++	+	+++
Cytochrome oxidase	−	−	+	+++	+	+++	+	+++	+	+	++
Malic dehydrogenase	+++	+	+	++	+	++	+	+	+	++	++
DPNH-diaphorase	++	±	+++	++	+++	++	+++	++	++	+	+++

PCT = Proximal convoluted tubule
DT = Distal tubule
PT = Proximal tubule
LH = Loop of Henle
CD = Collecting duct
Glom = Glomerulus.

Reaction is denoted by an arbitrary scale from − to ++++.

either lability to formalin fixation or of enzyme specificity, since rat tissues gave good results with the same medium. The method of Fishman and Baker (1956) for this enzyme is not specific (Janigan and Pearse, 1962). We were thus unable to obtain reliable results for this enzyme, and the results in transplants will not therefore be discussed.

Succinic dehydrogenase.—The reactivity of distal tubules in aglomerular and glomerular cortex is slightly greater than in the proximal convoluted tubules; the proximal tubule in the labyrinth cortex is, however, of equal reactivity to the distal tubule. No glomerular reaction was noted. In all cells the reaction product was diffuse throughout the cell (Fig. 2).

Malic dehydrogenase.—The reaction generally obtained was faint using " MTT " or " Nitro BT " even after 60 min. incubation, although the medium gave good results with rat kidney. The difference in reaction when compared with succinic dehydrogenase is puzzling in view of the relative QO_2 of the 2 enzymes in homogenates (Tyler *et al.*, 1964). Reaction was heaviest in the distal tubule throughout the cortex. Staining of arterial walls was prominent and of glomerular cells (Fig. 3).

Cytochrome oxidase.—The reactivity was diffuse throughout the cells. Distal tubule reactivity was most prominent in all parts of the cortex, although a good reaction was obtained in all parts of the proximal tubule (Fig. 4, 5). Glomeruli were almost free of activity.

DPNH-diaphorase.—Proximal convoluted tubules are slightly more active than distal tubules in all cortical areas; while the activity of the proximal tubule in the medullary rays is slightly less than that of proximal convoluted tubule. In one specimen the collecting ducts showed very intense activity.

The use of oil Red-O demonstrated the presence of considerable amounts of

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EXPLANATION OF PLATES

FIG. 1.—Frozen section of normal dog kidney cortex stained for acid phosphatase. ×160.

FIG. 2.—Frozen section of normal dog kidney cortex stained for succinic dehydrogenase. ×60.

FIG. 3.—Frozen section of normal dog kidney cortex stained for malic dehydrogenase using the " MTT " method. ×60.

FIG. 4.—Frozen section of normal dog kidney cortex stained for cytochrome oxidase. ×480.

FIG. 5.—Frozen section of normal dog kidney cortex stained for cytochrome oxidase. ×240.

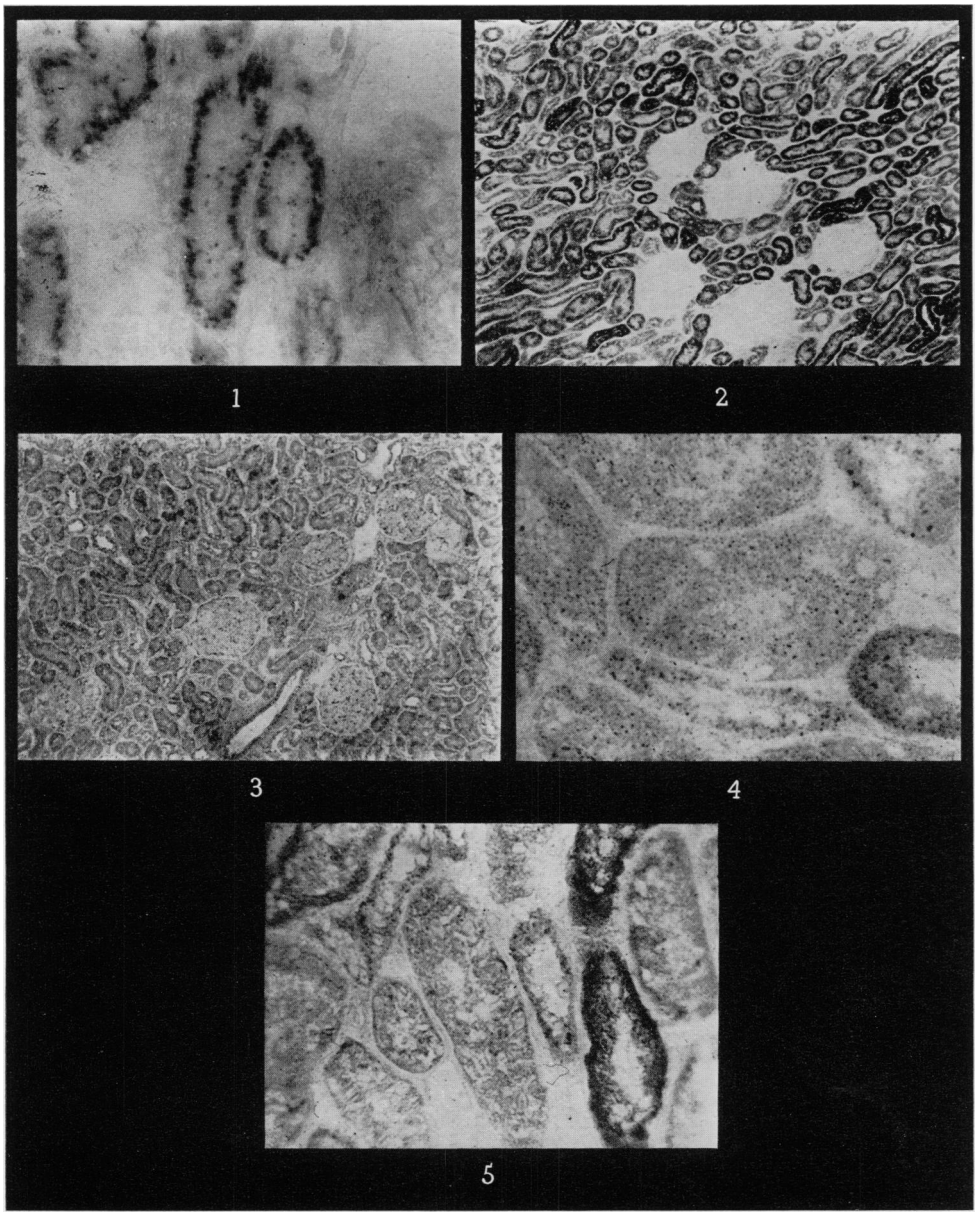
FIG. 6.—Frozen section of cortex removed from an oliguric homotransplanted dog kidney, stained for acid phosphatase. ×160. There is clumping and perinuclear aggregation of the reaction product.

FIG. 7.—Frozen section of cortex removed from an oliguric homotransplanted dog kidney, stained for acid phosphatase. ×60. Redistribution of the reaction product is again demonstrated in the kidney tubules, which are separated by oedema. The infiltrating cells show acid phosphatase activity.

FIG. 8.—Frozen section of cortex removed from an oliguric homotransplanted dog kidney, stained for succinic dehydrogenase. ×60. There is loss of activity in the proximal and proximal convoluted tubules, and little or no activity in the infiltrating cells. The tubules are grossly separated by oedema.

FIG. 9.—Frozen section of cortex removed from an oliguric homotransplanted dog kidney, stained for malic dehydrogenase. ×60. There is loss of activity in the proximal and proximal convoluted tubules; the infiltrating cells show some activity. The tubules are again separated by oedema.

FIG. 10.—Frozen section of cortex removed from an oliguric homotransplanted dog kidney stained for cytochrome oxidase ×480. The tubular epithelium is flattened and the tubules are separated by oedema. The infiltrating cells show some activity.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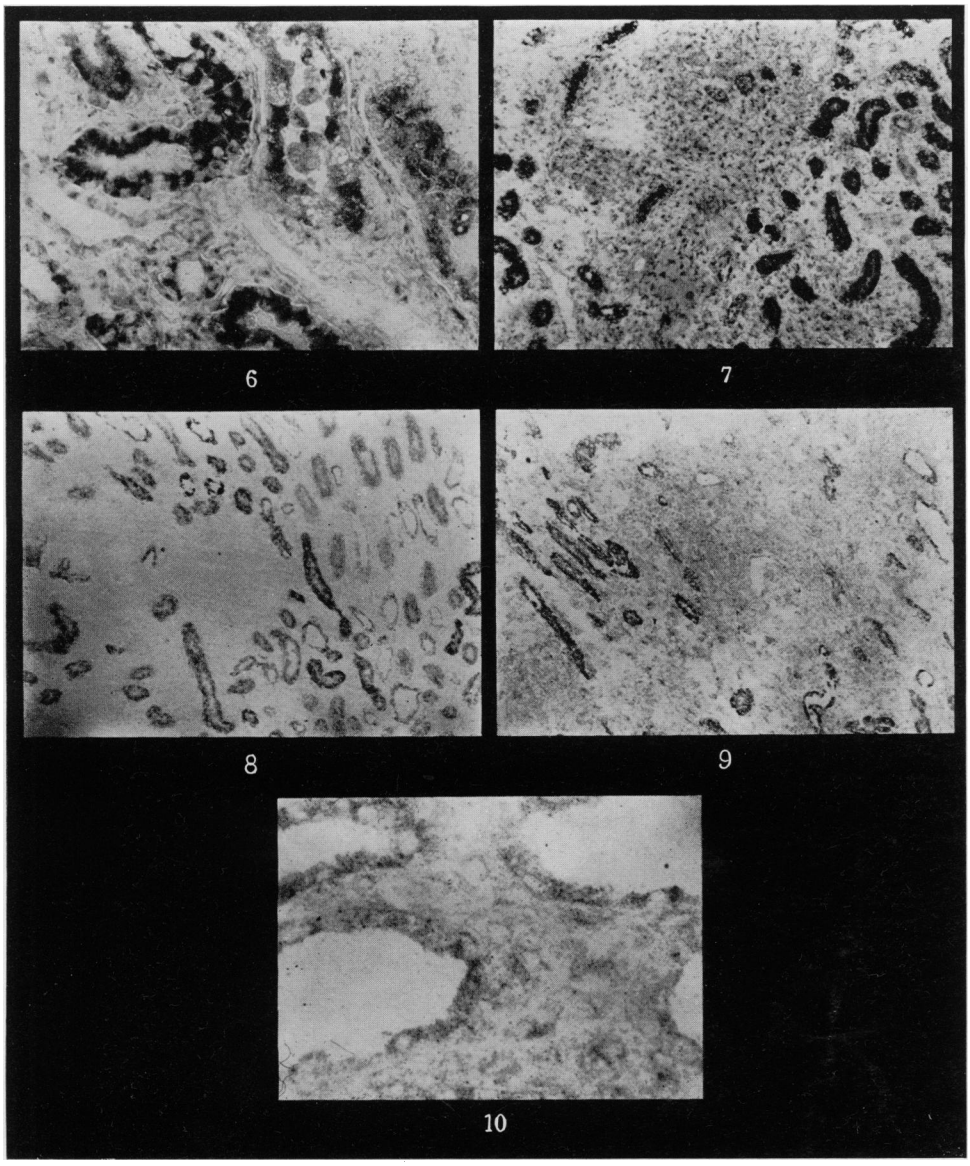

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neutral lipid occurring as large droplets in the proximal tubules and collecting ducts of the medullary rays. The lipid was also easily visualised in unstained sections with the interference phase microscope. Since the reaction product of many of the histochemical methods is soluble in lipids, care was taken not to identify such an occurrence in large lipid droplets as a pathological change.

Results of studies on transplanted kidneys

Autotransplants.—Differences between autotransplants and normal kidneys were small. Only in the proximal convoluted tubules were changes observed. Occasional proximal convoluted tubules showed a decrease in acid phosphatase and irregular localisation ; other tubules, normal in serial haematoxylin and eosin stained sections, showed " clumping " and perinuclear aggregation of droplets. These tubules showed decreased succinic dehydrogenase, DPNH diaphorase, and malic dehydrogenase activity. Oil Red-O stains showed the presence of small deposits of sudanophilic material in occasional degenerating proximal tubule cells. Very few infiltrating cells were seen in 2 of the kidneys. These were neutrophilic polymorphs, and showed the presence of the 2 hydrolases and small amounts of malic dehydrogenase.

Functioning homotransplants.—Renal tubules showed small changes similar to those occurring in autotransplants. No widespread damage was seen. A slight cellular infiltration was seen in the interstitium of the transplants. The cells composing this infiltration contained marked acid phosphatase and β-glucosaminidase activity, and faint malic dehydrogenase activity.

Oliguric homotransplants.—Because of interstitial oedema and cellular infiltration renal tubules showed a decrease in number per field and a flattening of the epithelial cells. There was a striking redistribution of the acid phosphatase droplets within the cells particularly in the straight portions of the proximal tubule. Proximal convoluted tubules also showed the redistribution which consisted of " clumping " and perinuclear aggregation of the product (Fig. 6, 7). Focal losses of activity were observed in some tubules and some had enzymically active debris in the tubule lumen. β-glucosaminidase showed some clumping of activity in those tubules where activity remained. Proximal tubules and proximal convoluted tubules (to a lesser extent), showed most loss. Cytochrome oxidase, DPNH diaphorase, succinic dehydrogenase and malic dehydrogenase all showed features in common, consisting of loss of enzyme activity in the proximal tubule and in the proximal convoluted tubule with conspicuously less change in the distal tubule (Fig. 8, 9, 10). Glomeruli and large blood vessels showed no changes in enzyme activity.

The interstitial cellular infiltration, consisted in large part of cells which stained with pyronine, the population reaching massive proportions in kidneys surviving for more than a week. Acid phosphatase and β-glucosaminidase were present in considerable quantities in these cells and in the case of β-glucosaminidase the specific activity appeared to be higher than that of renal tubule cells. It is clear that in homotransplants with heavy infiltration, these cells provide the major source of β-glucosaminidase and probably other acid hydrolases. Demonstrations of the 4 respiratory enzymes again revealed that the infiltrating cells contained very small amounts of these enzymes. A faint reaction for malic dehydrogenase was obtained and, focally, a weak reaction for cytochrome oxidase ; neither reaction, however, approached the level of that seen in normal renal tubule cells (Fig. 8, 9, 10).

Toluidine blue stains, revealed β- or γ-metachromasia in many infiltrating cells. Many infiltrating cells gave a positive reaction for the PAS stain. It is possible therefore that these cells are the source of extra hexosamine noted in homotransplants. (Williams, Morton, Tyler and Dempster, 1964).

DISCUSSION

The results presented in this paper offer information of value in interpretation of the quantitative enzyme data (Tyler *et al.*, 1964) and in elucidating the mechanism of rejection of homotransplanted kidneys. The loss of enzyme activity immediately after transplantation is seen to be a focal process, confined mainly to isolated proximal tubules. Focal damage appears as "foamy" vacuolation, disruption of acid phosphatase granules, deposition of neutral lipid, or complete loss of all the enzymes studied.

Renal tubule cell enzyme activity in secreting homotransplants, generally showed little difference from those in autotransplants, although small differences in total activity would have been difficult to detect histochemically.

Oliguric homotransplants showed striking changes in total enzyme activity and intracellular distribution when compared to functioning transplants. Different segments of the mammalian nephron vary in their enzymatic activity (Wachstein, 1955; Sternberg, Farber and Dunlap, 1956; Kissane, 1961) and sensitivity to various types of nephrotoxic injury. It is a particular characteristic of the mouse, rat, dog and human kidney at least, that the proximal tubular cells show the earliest and severest changes, per unit time, to ischaemia after temporary occlusion of the blood supply (Emmel, 1940; Koletsky, 1954; Burwell, 1955; Gowing and Dexter, 1956; Wachstein and Meisel, 1957) the changes in enzyme activity paralleling the degree of structural alteration.

Similar observations in this study suggest that the mechanism of kidney homotransplant rejection in the dog is essentially ischaemic in nature. The overall pattern reveals that so far as the enzymes we have studied are concerned, total enzyme activity is decreased by the oliguric stage through necrosis of the proximal tubular epithelium and, to a lesser extent, the distal nephron segments. The apparently greater sensitivity of the proximal tubular cells to ischaemia might be related to the greater number of large mitochondria in these cells as compared with other segments. It should also be pointed out that, while the cytological and topographical changes in the acid phosphatase droplets is an early occurrence in cell damage from other causes as well as ischaemia, recent evidence from studies on mouse liver (Trump, Goldblatt and Stowell, 1962) indicate that changes in these organelles might be secondary to mitochondrial damage.

Histochemical studies of enzymes in the cellular infiltration of homotransplants show high hydrolase and low dehydrogenase activity. Taken together with estimates of the amount of infiltration (Williams *et al.*, 1964) these results explain the high hydrolase and low dehydrogenase activity of long surviving renal homotransplants (Tyler *et al.*, 1964).

Thus at oliguria, low dehydrogenase activity results from renal ischaemic necrosis plus dilution with enzymatically inactive cells. High hydrolase activities in these circumstances are obtained by the presence of many infiltrating cells containing a high specific activity of these enzymes, and thus obscuring any decrease in hydrolase activity in the renal tubular cells.

GENERAL DISCUSSION AND CONCLUSIONS

Autotransplants

The small differences in specific enzyme activities between normal and autotransplanted kidneys can be attributed only to the effects of the period of ischaemia (30–40 min.) and surgical trauma involved in transplantation. These effects manifest themselves by focal depression of certain enzymes, as found histochemically ; this is possibly due in part to total physical loss of enzymically active cell components. That this is a more important factor as far as quantitative enzymology is concerned, than the interstitial oedema that arises, is borne out by the increased per cent DNA in autotransplants—which itself is not due to an increased mitotic index. In any case, the main depression appears to occur 24 hr. postoperatively, after which the enzyme levels may return to near normal limits by about the 4th day.

Functioning homotransplants

The kidneys of this group are subjected to the same factors that influence the specific enzyme activities of autotransplants, as well as to any additional factors resulting from their homologous environment. Both the quantitative enzymology and the histochemistry indicate that those kidneys which were definitely functioning well when removed at the fourth day, were very similar enzymatically to autotransplants.

Although the mean enzyme activities of a group of functioning homotransplants removed between the second and fourth days were lower than the mean autotransplant levels, the results from the biopsy experiments lead us to suppose that the majority of these functioning kidneys would shortly have become oliguric. We postulate, therefore, that there is a pre-oliguric state characterised by low enzyme levels as measured quantitatively, while the kidneys remain essentially similar to autotransplants histochemically. The kidneys of this group were not characterised by a greater degree of oedema than is seen in autotransplants, so tissue dilution would not account for the observed enzyme levels. Nor would the small number of infiltrating cells present at this stage seriously affect the enzyme levels. The most likely explanation of these findings is that there is a progressive loss of enzymes from the kidney tubular cells which manifests itself quantitatively before the oliguric stage is reached. The finding of high levels of a number of enzymes in the urine from homotransplanted kidneys would support this explanation (Tyler and Williams, unpublished).

Oliguric homotransplants

This group of kidneys has been divided into two, the division being made at the sixth day, so as to exclude from the first group those relatively long-surviving kidneys which are usually characterised by a massive cellular infiltration. Kidneys which became oliguric within 6 days of transplantation possessed lower dehydrogenase and glycosidase levels. Striking changes were seen histochemically ; particularly marked in the proximal tubules was the redistribution of acid phosphatase and loss of β-glucosaminidase. The histochemical changes are consistent with ischaemic damage to the kidney (Emmel, 1940 ; Koletsky, 1954 ; Burwell, 1955 ; Gowing and Dexter, 1956 ; Wachstein and Meisel, 1957). Ischaemia leads to

increased membrane permeability which in turn causes release of lysosomal enzymes, mitochondrial changes and loss of soluble and particulate matter from the cells (de Duve and Beaufay, 1959 ; Dawkins, Judah and Rees, 1959)—thus accounting for the low enzyme levels. The primary cause of the low enzyme levels at oliguria is therefore thought to be tubular ischaemia, though the increasing oedema and plasma cell infiltration will also lead to apparent changes in enzyme levels. It is probable that the lesion described by Darmady, Dempster and Stranack (1955), and which is demonstrated by the fourth day, is caused by ischaemia.

Homotransplants which became oliguric after the 6th day were all characterised by a markedly low dehydrogenase level, while most of them contained glycosidase levels significantly greater than the mean normal level. From the histochemistry of these kidneys it was obvious that these additional quantitative changes occurred as a result of the massive cellular infiltration. The infiltrating cells contained very high hydrolase specific activity and weak respiratory enzyme activity. From a knowledge of the wet weight/dry weight ratio, and the percentage DNA of a number of these kidneys, it was possible to deduce that the infiltrating cells out-numbered kidney cells by at least 4:1. Thus, the enzyme figures obtained from homogenates of these kidneys cannot be regarded as representative of renal tissue.

Our conclusions from these experiments are that homotransplanted kidneys are able to recover, enzymatically, from the effects of transplantation *per se*. Enzyme levels usually recover to near normal limits for a period of about 4 days, after which the specific activities fall progressively during the functioning period, due to an advancing state of tubular ischaemia. The increasing oedema and plasma cell infiltration lead only to dilution artefacts as far as quantitative enzymology is concerned. The whole process is presumably speeded up in homotransplants which survive for short periods (up to 5 days) and such kidneys may have low enzyme levels throughout their functioning period.

No evidence has been established from our limited data, of a " biochemical lesion " as was originally envisaged by Dempster (1955) in terms similar to those used by Peters (1936, 1953). Indeed, the electron transport chain appears to remain intact throughout the survival period of a homotransplanted kidney. The major biochemical changes are, in our opinion, attributable to the effects of ischaemia alone.

These conclusions have recently received support from the work of Kountz, Williams, Williams, Kapros and Dempster (1963). These colleagues have shown that homograft rejection is initiated by an attack of presumably host plasma cells on the vascular endothelium of the intertubular capillaries of the kidney. This leads eventually to destruction of these vessels, blockage of the vascular areas, and hence to a reduced renal blood flow and inadequate perfusion of the tubules. The commencement of vascular changes can be seen with the electron microscope as early as 48 hr. after homotransplantation. The electron microscope studies have also confirmed our postulate (Tyler *et al.*, 1964) that some tubular shedding takes place (Kountz *et al.*, 1963).

Additional evidence against the concept of a biochemical lesion has come from the recent work of Tyler, Williams and Dempster (1964). These workers have studied the incorporation of ^{14}C from (^{14}C)-glucose, (^{14}C)-succinate and (^{14}C)-acetate, into the soluble intermediates of chopped kidney tissue. Such metabolic pathways as glycolysis, the tri-carboxylic acid cycle and transamination appear to be still intact even at the oliguric stage.

It would appear that, so far as the homotransplanted kidney is concerned, but possibly other tissues could be included as well, that the parenchyma plays little or no part in the host⇌graft struggle; the primary changes in a "first set" kidney are vascular (Kountz *et al.*, 1963). It is interesting to note that the histological changes in a "second set" can be wholly attributable to an acute severe vascular catastrophe (Dempster, 1953). A tentative explanation would be that vascular endothelium is the structure which is most highly antigenic, hence the endothelial cells appear to be the primary target of the invading host cells. Alternatively, the host plasma cells are attracted to the vascular endothelial cells because these are the first foreign cells they encounter. Both explanations may be in part true. It is remarkable that many plasma cells, not fixed to the vascular endothelium, appear to be making no effort to attack tubular cells.

SUMMARY

A quantitative and histochemical enzymatic investigation has been carried out on normal, autotransplanted and homotransplanted dog kidneys. Some quantitative chemical analyses have been carried out to assess possible dilution artefacts.

The histochemical studies consisted of the demonstration of acid phosphatase, β-glucosaminidase, succinic dehydrogenase, malic dehydrogenase, DPNH diaphorase and cytochrome oxidase.

Explanations of the quantitative changes in enzyme activity seen in renal transplant homogenates are put forward on the basis of the histochemistry results. These results indicate that destruction of renal homotransplants in the dog is associated with, and probably initiated by, an ischaemic process.

Recent data from electron microscope, blood flow, and metabolic studies of the homotransplanted dog kidney confirm our suggestion that the major biochemical changes are due to ischaemia.

The biochemical changes demonstrated appear to be secondary to an attack on the vascular endothelium of the kidney by invading host plasma cells.

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