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

I. Some Quantitative Assessments of Enzyme Activity

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The cause of functional arrest of "first-set" homotransplated kidneys has for some years been the subject of much investigation and considerable speculation.

Dempster (1953*a*) discussed the available evidence at that time and rejected a number of possible causes of arrest of function, with the exception of vascular spasm. Evidence was presented which would allow one only to infer that homotransplants are rejected by means of an acquired immunity on the part of the host. Further work by Dempster (1953*b*) however, led him to doubt that "first-set" renal homotransplants were rejected by an actively acquired immunity manifesting itself by arterial spasm. Arterial spasm was found to be a late phenomenon in the rejection process. Even the discovery of a morphological lesion in the neck of the proximal tubule did not offer an adequate explanation of the onset of anuria and it was concluded that "a biochemical lesion . . . occurs in the tubule cells and that this initiates vascular spasm and complete cortical ischaemia, thus causing the anuria which, in the homotransplanted kidney, leads to parenchymal lysis" (Darmady, Dempster and Stranack, 1955).

Of the reasons for supposing that the kidney is responsible for its own destruction, the most important was the absence of evidence for free circulating antibody in the serum of the host; and absence of histological evidence for the fixation of this type of antibody in the transplanted kidney (Dempster, 1955). A second reason was the difficulty in explaining the behaviour of the homo-autotransplant, (Simonsen, 1953; Dempster, 1955). A third reason lay in the interpretation of the origin of the plasma cell infiltration characteristic of the first set homotransplanted kidney. Dempster (1953*a*) and Simonsen (1953) both interpreted these cells as originating in the kidney and representing a reaction of that organ against its host and, in Simonsen's opinion, against host antibody. Thus there appeared to be no explanation of the rejection process in terms of a host reaction. Dempster (1955) ruled out such physical factors as increased interstitial pressure and vascular stretch due to oedema and, having no evidence for an immunological reaction on the part of the host suggested a biochemical lesion within the tubule cells as an explanation of anuria.

Since the studies described here were commenced, unequivocal evidence for the host origin of at least a portion of the pyroninophilic cell infiltration has been

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presented (Porter and Calne, 1960; Fowler and West, 1961; Dempster and Williams, 1963).

The present studies were commenced with the prime object of testing the postulate that a biochemical lesion was responsible for the destruction of the homotransplanted kidney. While the work was in progress it became clear that it was also necessary to examine if possible the effect of the presence of plasma cells on renal tubule biochemistry.

It was decided to commence the investigations with the measurement of certain enzyme activities and their distribution within the cell. Succinic and Malic dehydrogenase were chosen to give information about the integrity of the electron transport chain and because they are known to be affected in a number of types of renal damage (Wachstein and Meisel, 1954; Rodin and Crowson, 1962). A group of glycosidases were also selected for study as representative of essentially lysosomal enzymes. These were β-glucuronidase, β-galactosidase and β-glucosaminidase. Acid hydrolases are known to change total activity and intracellular distribution in various types of tissue damage including ischaemia (de Duve and Beaufay, 1959) action of antibody (Fennell and Santamaria, 1962) and sucrose nephrosis (Janigan and Santamaria, 1961). From the initial quantitative measurements it became obvious that a more detailed investigation involving histochemistry and analytical chemistry was necessary. These investigations are reported in the two succeeding papers. Finally, the quantitative enzymology was extended to a study of renal biopsy material from individual kidneys. This paper reports the quantitative enzymological data. A preliminary report of part of this work has already appeared (Williams, Tyler, Morton, Nemeth and Dempster, 1962).

MATERIALS AND METHODS

Animals.—Greyhound bitches of average weight 22 kg. were used throughout this investigation.

Surgical techniques.—Kidneys were transplanted by techniques previously described (Dempster 1953*a*, 1954).

Series 1: *Removal of kidneys.*—Functioning autotransplants were removed between the 2nd and 7th days following transplantation. Homotransplants were divided into 2 main groups—those removed between the 2nd and 4th days while still functioning, and those which proceeded to oliguria. Oliguric kidneys were further divided into 2 groups, according to the day on which they became oliguric. The first sub-group included kidneys becoming oliguric on the 4th, 5th or 6th day; the second sub-group includes kidneys becoming oliguric later than the 6th day. The longest survival was 17 days.

Series 2.—Wedge biopsies of wet weight about 0·5 g. were taken before transplantation and at various times during the life history of the transplant. The first biopsy was taken from the lower pole, the second from the upper pole and the third from the centre, in order to minimise the risk of polymorphonuclear cell infiltration in biopsy specimens. The biopsy sites were closed with 5/0 silk sutures. In no case were more than 3 biopsies taken from one kidney. In one dog a wedge biopsy was taken from both normal kidneys and the renal arteries clamped for 60 min. The circulation was then restored and the kidneys removed 24 hr. later. Most of the kidneys in series 2 were removed while still functioning, at times varying between 24 hr. and 9 days, but 2 proceeded to oliguria. Biopsies were frozen in liquid oxygen immediately after removal and stored at $-40°$ in sealed polythene bags. All biopsies from one kidney were then assayed on one occasion. We have previously shown that storage in this way for up to one month, does not affect the total activity of these enzymes in dog kidney (unpublished data). Fourteen dogs were used for the experiments in Series 2. In 6 dogs the experiment had to be prematurely terminated as, following the 24-hr. biopsy, some died apparently from the repeated anaesthetic and in others the transplant failed to resume function.

Assessment of oliguria.—Kidneys were judged to be truly oliguric when a sudden decrease in daily urine output occurred together with a poor response to successive infusions of 500 ml. of 0·9 per cent saline and 300 ml. of 2 per cent saline. This condition was often accompanied by a high blood urea level and a toxic syndrome (Dempster, 1953*c*). The serum trypsin-inhibitor test (Tyler, Lister Cheese, Struthers and Dempster, 1962) was also used on some occasions.

Preparation of homogenates.—Decapsulated cortex (about 0·5–1 g. wet weight) was quickly dissected from the kidney and suspended in 4–9 wt. vol. of ice-cold medium. After being finely chopped with scissors it was homogenised in a Potter-Elvehjem homogeniser (Potter and Elvehjem, 1936). Frozen material such as was used in series 2 was treated in a similar way after allowing the tissue to reach 0°.

Enzymology.—β-glucuronidase, β-galactosidase and β-glucosaminidase were estimated by hydrolysis of phenolphthalein glucuronide, *o*-nitrophenyl-β-D-galactoside and *p*-nitrophenyl-β-D-glucosaminide respectively (Findlay, Levvy and Marsh, 1958; Levvy and Marsh, 1959; Conchie, Findlay and Levvy, 1959). Malic and succinic dehydrogenases were estimated manometrically (Umbreit, Burris and Stauffer, 1957). In series 1 all enzymes were estimated on complete homogenates of kidney cortex, the media for homogenisation being water for the hydrolases and 0·25 M sucrose for the dehydrogenases. In series 2 all enzymes were estimated on the same water homogenate.

Dry weights.—The dry weight of kidney homogenate was determined by dialysing a known volume of homogenate against several changes of distilled water at 4° and transferring the non-diffusable residue to a previously weighed vessel before drying at 110° to a constant weight. Enzyme activities were expressed per milligram dry weight.

Histology.—Material for paraffin sections was taken immediately upon removal of the kidney and fixed in 10 per cent formol-saline. When serial biopsies were taken (series 2) a small portion of each was removed from the centre of the biopsy and fixed. Sections were stained with methyl-green-pyronine or haematoxylin and eosin.

RESULTS

The results of the two series of experiments will be presented separately. The results of the first series contain the pooled information derived from single enzyme measurements made on a number of normal, autotransplanted and homotransplanted kidneys. The second series contains quantitative enzyme measurements derived from serial biopsies from individual transplanted kidneys.

Series 1

It soon became obvious to us that the intention to examine possible changes in the intracellular distribution of the lysosomal enzymes could not be reliably carried out quantitatively. This was due to the great individual variability in the ease of homogenisation shown by the greyhound kidney, and also because of the variable degree of interstitial oedema which arises in transplanted kidneys. We have, therefore, measured the total glycosidase activity after homogenising in water. The results of series 1 are presented in Table I. Enzyme activities are referred to dialysed dry weight.

Autotransplants.—Each enzyme had a reduced mean specific activity between 75 per cent (succinic dehydrogenase), and 91 per cent (β-galactosidase) of normal. The differences shown by the two dehydrogenases and β-glucuronidase were significantly lower than normal ($P<0·05$).

Homotransplants.—(*a*) Functioning kidneys : The mean values of each enzyme showed further decreases in specific activity over those occurring in autotransplants. These further decreases were more pronounced in the dehydrogenases, these activities being significantly lower than in the autotransplants ($P<0·05$).

TABLE I.—*Mean Enzyme Levels of Normal and Transplanted Kidneys. Values are Expressed as Percentage of the Activity Shown by Normal Kidneys*

Enzyme	Normal	Functioning Autotransplants 2–6 days	Functioning Homotransplants 2–4 days	Oliguric Homotransplants 3–6 days	Oliguric Homotransplants 7–17 days
Malic dehydrogenase	100 (82·1±22·5)	82·6	55·0	63·2	46·3
Succinic dehydrogenase	100 (69·0±20·5)	75·4	51·6	57·8	44·7
β-Glucuronidase	100 (11·4±1·5)	87·7	72·8	75·4	121·0
β-Galactosidase	100 (11·5±2·0)	91·3	73·9	79·1	141·0
β-Glucosaminidase	100 (340±36)	82·6	67·9	76·2	116·0
No. of kidneys examined	15	10	8	8	4

Figures in parentheses are actual enzyme units with standard deviation.
Dehydrogenases—μl. O_2 absorbed/mg./hr.
Glycosidases—μg. aglycone released/mg./hr.

(*b*) Oliguric (3rd–6th day): Although the means indicated a slight rise in specific activity when compared with the previous group, the differences were not significant by Students " t " test.

(*c*) Oliguric (7th–17th day): All kidneys in this small group showed striking increases in glycosidase activities to more than 100 per cent of normal values; dehydrogenases on the other hand showed further decreases.

Series 2

Due to technical difficulties in obtaining more than one relatively large biopsy from any one kidney, the data is not as complete as was originally intended. β-glucuronidase estimations were often omitted owing to insufficiency of tissue.

Enzymology.—Data from 11 homotransplanted (and two autotransplanted) kidneys are presented in tables II, III and IV. Also included in table II are the results obtained from the 2 normal kidneys which had the renal artery clamped *in situ* for one hour before restoration of blood supply for 24 hr. The results are

TABLE II.—*Enzyme Levels Obtained From Kidneys Biopsied Immediately Before, and 24 Hr. Following Transplantation. Two Control Kidneys were Ischaemic for* 1 *Hr.* in situ, *and were Removed 24 Hr. After Restoration of Blood Supply*

Kidney		Zero time (actual enzyme units) β-glucuronidase	Zero time (actual enzyme units) β-glucosaminidase	Zero time (actual enzyme units) Succinic dehydrogenase	24 hr. post-operative (per cent of value at zero time) β-glucuronidase	24 hr. post-operative (per cent of value at zero time) β-glucosaminidase	24 hr. post-operative (per cent of value at zero time) Succinic dehydrogenase
Homotransplant	1	—	453	129	—	67	77
,,	3	—	363	99	—	107	111
,,	6	—	402	136	—	101	82
,,	7	6·5	337	110	78	53	84
,,	8	11·2	295	135	81	67	68
Autotransplant	2	7·6	285	85	92	90	83
Control	1	6·5	221	98	99	92	101
,,	2	7·2	305	103	96	102	97

expressed as percentages of the specific activities at zero time. The actual enzyme activities of the kidneys at zero time are expressed in appropriate enzyme units (Williams *et al.*, 1962). The results suggest that within 24 hr. of transplantation enzyme levels may be depressed (Tables II, III). From the limited data in Table III it appears that with the exception of one homotransplant (H4), there was no further decline in enzyme levels between 24 and 96 hr., following transplantation. Indeed there is evidence for an improvement in enzyme levels in some cases.

TABLE III.—*Enzyme Levels Obtained From Kidneys Biopsied Immediately Before, then 24, 72 and 96 Hr. Following Transplantation*

	Zero time (actual enzyme units)			24 hr. Postoperative (per cent of value at zero time)			72 hr. Postoperative (per cent of value at zero time)			96 hr. Postoperative (per cent of value at zero time)		
Kidney	*a	b	c	a	b	c	a	b	c	a	b	c
Homotransplant 2	—	435	118	—	100	96	—	129	112	—	97	100
,, 4	—	384	113	—	85	110	—	—	115	—	83	70
,, 5	—	334	94	—	83	83	—	103	138	—	88	114
Autotransplant 1	8·7	327	111	96	95	64	79	84	88	83	102	88

* a = β-glucuronidase b = β-glucosaminidase c = succinic dehydrogenase

The kidney H4 showed a marked drop in succinic dehydrogenase between 72 and 96 hr., and this suggests that it may have become oliguric about the 5th or 6th day after transplantation. Data from H10 which was oliguric on the fifth day is shown in Table III and the 3 enzymes studied show a marked depression at this point. The kidney H9 had normal enzyme levels on day 5, but each enzyme was depressed (succinic dehydrogenase markedly so) on day 9 when the kidney was still secreting well. H11, which did not become oliguric until the 12th day, had depressed enzyme activity on day 7. It was not possible to distinguish enzymatically between autotransplants and homotransplants, when studied within the first three days.

Histology.—Histological examination of biopsies with haematoxylin and eosin or methyl-green-pyronine stains revealed the usual homotransplant histology (Dempster, 1953*a*). Previous biopsy sites were sometimes associated with local

TABLE IV.—*Enzyme Levels Obtained From Kidneys Biopsied Immediately Before, then After the 4th Day Following Homotransplantation*

	Zero time (Actual enzyme units)			5th Day (per cent of value at Zero time)			7th Day (per cent of value at Zero time)			9th Day (per cent of value at Zero time)			12th Day (per cent of value at Zero time)		
Kidney	*a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Homotransplant 9	12·5	588	110	97	88	99	—	—	—	88 (Functioning)	70	59			
,, 10	10·1	286	93	81 (Oliguric)	76	59									
,, 11	8·8	360	103	—	—	—	78 (Functioning)	82	67	—	—	—	73 (Oliguric)	85	37

* a = β-glucuronidase b = β-glucosaminidase c = succinic dehydrogenase

polymorphonuclear cell infiltration, but the biopsies used for enzymology were essentially free of these cells.

DISCUSSION

The results from Series 1 indicated that the enzyme levels of transplanted kidneys were lower than normal when expressed in terms of dry-weight. That this is partly due to the effects of temporary ischaemia and surgery is suggested by the data from autotransplants. More significant was the fact that lower enzyme levels were observed in functioning and oliguric homotransplants (removed within six days of transplantation) than were found in autotransplants. This suggests a fundamental difference between auto- and homotransplants which can be expressed in terms of some enzyme specific activities. No differences were observed between functioning and oliguric homotransplants removed within 6 days, although we had no indication as to when the kidneys in the functioning group would have become oliguric. Three out of 8 of these functioning kidneys possessed dehydrogenase activities within the autotransplant range, and it may be that the remaining 5 which had low enzyme levels, would shortly have become oliguric. The problem remained, therefore, as to whether the enzyme activities declined soon after homotransplantation, or near oliguria. The results from Series 2 indicate that a kidney (whether an autotransplant, homotransplant, or an *in situ* control) may have depressed enzyme levels as a result of a period of ischaemia. This depression of enzyme levels appears to be temporary and with the exception of one kidney there was no further decline in enzyme levels between 24 and 96 hr. following homotransplantation. This may seem at variance with the data from Series 1 ; however, each of the " functioning kidneys " in this series were transplanted to the neck in contrast to the " oliguric kidneys " which were, in the main, pelvic transplants. From Dempster's earlier work it is known that the majority of kidneys homotransplanted to the neck are rejected within the first 5 days (Dempster, 1953*a*). If we accept that the majority of functioning kidneys in Series 1 were soon to be rejected, the combined data of Series 1 and 2 indicate that apart from a transient fall at 24 hr., the specific enzyme activities of homotransplants do not decline until after the 4th day, unless rejection occurs within or shortly after this period.

Homotransplants surviving longer than 6 days are characterised by a low dehydrogenase activity irrespective of whether the kidneys are functioning or oliguric. The glycosidase activity of these kidneys, on the other hand, appears to increase to above normal levels.

The results indicate that dehydrogenase activity falls progressively after the 4th postoperative day. Glycosidase activity probably follows a similar curve up to the first week, thereafter it increases with the time of survival of the homotransplant. Before the significance of these changes in enzyme specific activities can be discussed, in terms of renal function, it becomes necessary to consider possible causes, bearing in mind that all the activities are calculated on the basis of dialysed dry weight. Such factors as interstitial oedema and cellular infiltration, need first to be assessed as these may affect the specific enzyme activities by dilution of renal tissue. These factors have been investigated and the results are presented in the 2 succeeding papers. The results of the quantitative enzymology will be discussed further in the light of these other investigations.

SUMMARY

The respiratory enzymes succinic dehydrogenase and malic dehydrogenase, and the glycosidic enzymes β-glucuronidase, β-galactosidase and β-glucosaminidase have been measured quantitatively in whole homogenates of normal, autotransplanted and homotransplanted dog kidneys.

Some of the above enzymes have been measured in serial biopsies removed before and at various times following transplantation. The effect of a 1 hr. period of ischaemia in normal kidneys was studied in the same way.

The effects of ischaemia and surgery may lead to depressed enzyme levels at 24 hr.

Kidneys not undergoing early rejection are able to recover to or above their preoperative enzyme levels within the next 48–72 hr.

Homotransplants becoming oliguric within the first 5 days may have depressed enzyme levels during the functioning period.

Homotransplanted kidneys surviving longer than the 5th day have essentially normal enzyme levels at this point. Thereafter there is a progressive decrease in the level of enzymes—particularly of succinic dehydrogenase. Glycosidase activity may rise to above normal levels in long surviving homotransplants.

Before the significance of the enzyme changes can be discussed it is necessary to assess the effects of such factors as interstitial oedema and foreign cell infiltration.

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