ENZYME CHANGES IN EXPERIMENTAL RENAL MICROCYSTIC DISEASE

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Summary.—In an experimental model of human microcystic disease in rats, abnormal changes in tubular enzyme activity were detected as the lesion developed. Decreased activity for alkaline phosphatase, Mg++-ATPase, and Na+K+-ATPase were detected mainly in the proximal tubule, where the lesion was most pronounced. Areas of early fibrosis between the cystic tubules were associated with acid phosphatase activity.

In a previous report (McGeoch, Woodhouse and Darmady, 1972), renal changes in rats were described as mimicking infantile polycystic or microcystic disease. In view of the complexity of the terminology in the literature, it has been decided to restrict the name of this condition to microcystic disease. The kidney lesion has been alternatively named "the nephrotic syndrome of early infancy" (Giles et al., 1957), "infantile nephrosis" (Fetterman and Feldman, 1960), "congenital nephrotic syndrome" (Hallman, Norio and Kouvalainen, 1967) and "infantile polycystic disease" (Darmady, Offer and Woodhouse, 1970).

In cases of human microcystic disease, changes in the enzyme alkaline phosphatase have been reported in kidney tubules by Giles et al. (1957) and Fetterman and Feldman (1960). The tubular lesion of microcystic disease has been studied by electron microscopy by Hjelt, Stjernvall and Hallman (1959) and abnormal changes in the plasma membrane brush border of the tubule epithelial cells were noted. Many enzymes, including alkaline phosphatase, are localized at the plasma membranes and the brush border (Schmidt and Dubach, 1972; Heidrich et al., 1972). A relationship between the morphological lesion and the change in enzyme activity may be postulated.

In the experimental model of human microcystic disease a proximal tubule lesion was produced with membrane changes similar to those in human microcystic disease. Using the model the activities of alkaline phosphatase, Mg++ ATPase, Na+ K+-ATPase and acid phosphatase were assayed as the hyperplastic dilatation of the kidney tubules developed.

MATERIALS AND METHODS

The rats used were 5-week old Allen & Hanbury Wistar–Sprague–Dawley strain. The method of inducing the lesion was as previously described (McGeoch et al., 1972). The rats were in 9 groups. The first 5 groups were dosed for 7 weeks with the anti-inflammatory compound 5, 6, 7, 8 tetrahydrocarbazole-3-acetic acid (AH2835) and sacrificed at 0, 4, 5, 6 and 7 weeks. Each of these 5 groups contained 1 control and 2 test rats of each sex. The 4 latter groups were dosed for 6 weeks and sacrificed at 9, 12, 15 and 18 weeks. They con-
tained 2 control and 5 test rats for each sex per group. The 4 latter groups were thus observed over a 12-week recovery period.

Sacrifice was by decapitation and the kidneys removed without delay. The left was placed in frozen carbon dioxide and used for histochemistry and the right, of the male rats only, was placed in iced 3 × 10⁻² mol/l Tris buffer pH 7-6 for biochemical assay. Portions of each kidney for male and female rats were retained for routine histology, microdissection and electron microscopy.

From the left kidney 8 μm cryostat sections were fixed in 4% formol calcium at -2 to -3°C for 2 min, washed in cold distilled water and the following assays were performed:

1) The Wachstein and Miesel ATPase assay (1957) using the Gomori lead nitrate method at pH 7-2, incubation time 2 hours. The inhibition of the ATPase by lead was reduced by filtration of the media before incubation. (2) The calcium phosphate method for alkaline phosphatase, incubation time 15 min (from Gomori, 1952; Lilie, 1954). (3) The lead nitrate method for acid phosphatase, incubation time 15 min (modified from Gomori, 1950).

All incubations were carried out at 37°C and for each assay substrate and enzyme controls were performed. The inhibitor used in the enzyme controls was 2% sodium azide for 30 min after fixation.

The right kidney was divided into outer and inner cortex, weighed, homogenized in cold 3 × 10⁻² mol/l Tris buffer, pH 7-6, diluted and stored at -20°C. The activities of ATPase's were determined in 3 different incubation media after a modified method of Gyorvay and Kinne (1971). Between 30 and 1000 μg wet kidney homogenate was present in each incubation medium. The wide range was necessary as test rat kidneys at Weeks 6 and 7 were very oedematous, with low enzyme levels. All media contained 3 × 10⁻² mol/l Tris buffer, 3 × 10⁻³ mol/l Tris ATP and 6 × 10⁻² mol/l MgSO₄. Two of these media (Media 2 and 3) also contained 2 × 10⁻² mol/l KCl and 1 × 10⁻¹ mol/l NaCl; to Media 3, 2 × 10⁻³ mol/l ouabain was added. For each experiment 2 control determinations of the 3 media were included, one without the kidney homogenate and the other without the ATP.

After 30 min incubation at 37°C the reaction was stopped in ice, 1 ml of cold 20% T.C.A. was added and left for 10 min. The media were centrifuged at 2000 rev/min and a micro-determination for phosphate performed on aliquots of the supernatant after the method of Baginski and Zak (1960). The mean of the activity found in incubation mixtures 1 and 3 was taken as the Mg⁺⁺-ATPase; the difference between incubation mixture 2 and the Mg⁺⁺ ATPase was taken to be the sodium and potassium stimulated, ouabain-inhibited ATPase (Na⁺⁺K⁺⁺-ATPase). The protein concentrations of the homogenates were determined according to the method of Lowry et al. (1951) with bovine serum albumin used as a standard. The enzyme activity was expressed as μmol phosphorus produced per mg of protein in 30 min (μmol/mg protein/30 min).

Each assay was performed in duplicate and the mean taken. The results for each group represent the values of 1 control rat and the mean of 2 test rats for each of the outer and inner cortical homogenates.

In addition, the in vitro effect of AH2835 on the activities of Mg⁺⁺-ATPase and Na⁺⁺K⁺⁺-ATPase was tested by incubating 4 test and 6 control rat kidney homogenates with 10 ng of AH2835 in the incubation media; 10 ng AH2835 is the equivalent dose for 333-3 μg kidney for dosing at 30 mg/kg.

RESULTS

Cystic dilatation and hyperplasia of the tubules followed the same pattern of development as previously reported (McGeoch et al., 1972). On cessation of dosing the rats with AH2835, the hyperplasia in the proximal and distal tubules continued until Week 9 but the intercellular and extracellular oedema was no longer present. Interstitial round cell infiltration increased and by Week 18 small patches of fibrosis were situated in the outer cortex. Some of the dilated proximal tubules had degenerative changes and diverticula. A few of the glomeruli showed abnormal changes, with thickening of the basement membrane and proliferation of the endothelium, epithelium and mesangial cells. Further details of the morphological changes will be the subject of another communication.
**Histochemical results**

Histological localization of ATPase in control rat kidneys occurred at the luminal membrane and the basal infoldings of the plasma membrane of the proximal and distal tubules. It was also localized in the basal membranes of the thin limbs and collecting ducts, in the epithelium of the glomerular capillary loops and throughout the blood vessels. The ATPase activity is that of Mg\(^{++}\)-ATPase, the Na\(^{+}\)K\(^{+}\)-ATPase being inhibited by the concentration of lead in the Wachstein and Miesel assay (Novikoff et al., 1961; Tormy, 1966; Jacobsen and Jørgensen, 1969). Alkaline phosphatase was demonstrated at the luminal brush border membrane of the proximal tubules and acid phosphatase as intracellular granules towards the basal side of the proximal tubule cells.

All changes in enzyme activity affected male rats more than female rats and the superficial nephrons (SN) of the outer cortex more than the juxtamedullary nephrons (JN) of the inner cortex. All enzyme changes were most pronounced in the pars convoluta of the proximal tubules and to a lesser extent in the distal tubules. ATPase reduction in the thin limbs, collecting ducts, glomeruli and

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![Graph showing enzyme activity changes over weeks of experiment.](image)

**Fig. 1.**—Histochemical localization of ATPase in pars convoluta, luminal cell border.
EXPLANATION OF PLATES

The plates illustrate the histochemical localization of ATPase, alkaline phosphatase and acid phosphatase activity in the superficial nephrons of male rats.

Fig. 3.—ATPase localization in a test (3a) and control (3b) rat kidney after 7 weeks dosing with AH2835. No ATPase activity is demonstrated at the luminal membrane of the proximal tubule of the test animal. ×170.

Fig. 4.—ATPase localization in a test rat at Week 18 of the experiment. Cystic areas lacking enzyme activity persist in the outer cortex. ×50.

Fig. 6.—Alkaline phosphatase localization in a test (6a) and control (6b) rat kidney after 7 weeks dosing with AH2835. There is complete loss of alkaline phosphatase activity from the proximal tubules in the test rat. ×170.

Fig. 7.—Alkaline phosphatase localization in a test rat at Week 18 of the experiment. Persisting cystic tubules lack alkaline phosphatase activity at the peripheral cortex. ×50.

Fig. 9.—Acid phosphatase localization in test (9a) and control (9b) rat kidney after 7 weeks dosing with AH2835. A large cystic tubule to the right of Fig. 9a lacks enzyme activity; less affected tubules show activity towards the luminal side of the tubule cells (arrow). In the control (9b) activity is localized towards the basal side of the cells (arrows). ×170.

Fig. 10.—Acid phosphatase localization in a test rat at Week 9 of the experiment. The arrow indicates an area of round cell infiltration, below which tubules possess high acid phosphatase activity. ×50.
McGeoch and Darmady
blood vessels was minimal. Histochemical enzyme activity is denoted by + signs and represents an overall degree of intensity of localization within the particular cortical area. Each histogram section represents the mean localization for all animals in that group.

Figures 1 and 2 show a decrease in ATPase activity in the proximal tubules up to 6 weeks of dosing with AH2835 in male rats. The effect occurred in the SN more than the JN and at the luminal membrane more than the basal membrane (Fig. 3).

On cessation of dosing, the test values approached those of the controls by Week 18, except in the SN, where dilated cystic tubules persisted (Fig. 4). The return of ATPase activity occurred more quickly at the basal end of the plasma membrane than the luminal membrane.

Figure 5 shows that changes in alkaline phosphatase in male rats at the luminal membrane of the proximal tubules were more pronounced than those for ATPase. By Week 6 and 7 of dosing no activity could be demonstrated in

![Graph showing enzyme activity over weeks](image)
the pars convoluta of the SN (Fig. 6). On cessation of dosing the return of activity to that of the controls was again less than ATPase activity (Fig. 7).

Acid phosphatase reduction was minimal (Fig. 8, male rats), the enzyme activity sites tending to redistribute to less cystic portions of the proximal tubules by Week 6 and 7 (Fig. 9): at Week 4 of dosing an increase in the SN occurred. On cessation of dosing areas of early fibrosis occurred between the cystic tubules associated with the acid phosphatase activity (Fig. 10).

**Biochemical results**

Changes in ATPase's detected by biochemical assay were similar to those detected by histochemistry. The enzyme activities for the controls were higher at the beginning of the experiment than at the end, suggesting higher levels in immature 5-week old rats.

Changes to the Mg^{++}-ATPase and Na^{+}K^{+}-ATPase occurred in the outer cortical homogenates more than the inner cortical homogenates and affected the former enzyme most (Fig. 11 and 12). The activities decreased while the
rats were dosed with AH2835 and on cessation of dosing increased, in most cases above that of the controls. The Na+K+-ATPase of the outer cortex showed the greatest increase.

In addition, there was a direct inhibiting effect of AH2835 on Mg++-ATPase and Na+K+-ATPase (Fig. 13). This occurred in 6 control homogenates and 2 out of 4 test homogenates incubated in vitro with AH2835. By comparing the percentage decrease in ATPase activity of test from control kidney homogenates, it is seen that the direct inhibition by AH2835 in vitro is less than when the drug is administered in vivo to the rats. The percentage decrease of Mg++-ATPase for Week 7 control rat outer cortical homogenate incubated with 10 ng AH2835 was 31.63%, while that for the test Week 7 rats from control was 75.47%. When Week 7 test rat outer and inner cortical homogenates were incubated in vitro with AH2835, little change in the enzyme activity occurred. Tests performed on Week 6 homogenates and Week 9 homogenates produced a decrease in Mg++-ATPase and Na+K+-ATPase.

**DISCUSSION**

In the experimental model of microcystic renal disease, referred to for the purpose of the discussion as (M), the morphological abnormalities in the kidney
tubules were correlated with enzyme changes in the tubules. The hyperplasia and dilatation of the tubular lesion developed over the period of dosing with AH2835, affecting male rats more than female rats and the SN more than the JN (M). The enzyme changes were seen to increase as dosing proceeded and affected males more than female rats and the SN more than the JN. The pars convoluta of the proximal tubule had the greatest degree of cystic dilatation and hyperplasia, and there the loss of enzyme activity by 6 or 7 weeks' dosing was shown by histochemistry to be greater than other nephron segments. In the proximal and distal tubules changes in the membranes were detected by electron microscopy, affecting the luminal membrane more than the infoldings of the basal plasma membrane (M). Similarly, the decrease in enzyme activity affected enzymes associated particularly with the luminal membrane, the alkaline phosphatase and the Mg$^{2+}$-ATPase (Heidrich et al., 1972; Kinne, Schmitz and Kinne-Saffran, 1971) more than the Na$^+$-K$^+$-ATPase particularly associated with the basal membrane (Schmidt and Dubach, 1971). Mg$^{2+}$-ATPase is also associated with the whole enveloping plasma membrane.

Small degenerative changes in the rat kidneys at Week 4 (a period of high

![Graph](image-url)
proteinuria (M) could have stimulated an increased lysosomal activity accounting for the increased acid phosphatase activity at that period.

The in vitro biochemical incubation of homogenates with AH2835 demonstrated that the drug directly inhibited Mg++-ATPase and Na+K+-ATPase. There was some evidence to suggest that AH2835 accumulated in the kidney. The percentage decrease of ATPase activity was greater in rats that had received AH2835 in vivo, comparing test and control values, than that produced when AH2835 was incubated in vitro with the control rat homogenates. Also, there was no further in vitro inhibition by AH2835 on the ATPase activity of test rat homogenates that had been dosed the longest period of 7 weeks. Spot tests (Feigl, 1956) for carbazole derivatives performed on test and control homogenates gave positive results for test animals at Week 6 and 7. It is postulated that the drug, by Week 7 of dosing, has accumulated in the kidney to a sufficient level to saturate the sites available for inhibitors of ATPase activity. The inhibiting effect of anti-inflammatory compounds on Na+K+-ATPase activity has been documented (Iwasaki and Aizawa, 1969).

If the loss of ATPase activity were due entirely to direct inhibition by AH2835 accumulating in the kidney, a return of activity level to that of the controls would be expected by Week 18, a 12-week recovery period being adequate for removal of the drug from the kidney. However, many cystic tubules in the outer cortex at Week 18 showed no histochemical ATPase activity. It is concluded that AH2835 induced a hyperplastic cystic change to the proximal tubules, the membranes of which were damaged as the lesion progressed and enzyme
activity localized at the membranes was lost. Also, the drug may have accumulated in the tubular cells exerting a direct inhibition on the ATPase’s.

In rats dosed with AH2835 symptoms of polyuria and loss of electrolytes in the urine occurred (Poynter, Selway and Spurling, 1968). These features may be correlated with the loss of Na⁺K⁺-ATPase activity in the kidney, an enzyme shown to have a close relationship with the Na⁺K⁺ exchange pump handling the electrolyte transport in the kidney (Katz and Epstein, 1967). Associations between the Mg⁺⁺-ATPase (Parkinson and Radde, 1971) and alkaline phosphatase (Schmidt and Dubach, 1969) and exchange of electrolyte transport in the kidney have been postulated. The abnormally high levels of electrolytes in the urine disappeared in the rats when dosing with AH2835 ceased, and at the same time alkaline phosphatase, Mg⁺⁺-ATPase and Na⁺K⁺-ATPase increased towards that of the controls, indicating a possible correlation between enzyme decrease and loss of electrolyte transport properties of the kidney. A similar correlation in a human case of microcystic disease has been recorded only once by Giles et al. (1957), with low levels of alkaline phosphatase in the cortical tubules, polyuria and hypokalaemia in the advanced stages of the nephrotic syndrome.

To conclude, the changes in alkaline phosphatase activity reported in human cases of microcystic disease were also detected in an experimental model of the disease in rats. In addition, changes in Mg⁺⁺-ATPase, Na⁺K⁺-ATPase and acid phosphatase were produced in the model. The severity of the enzyme changes appears to be correlated with the severity of the morphological and physiological changes of the lesion.

It is hoped in the future that studies of this nature will be performed in renal biopsies of human cases and contribute to the understanding and aetiology of the disease process.

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REFERENCES


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