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THE ROLE OF CARBONIC ANHYDRASE IN RENAL REABSORPTION OF BICARBONATE

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It is now generally accepted that part at least of the bicarbonate filtered by the glomeruli is reabsorbed by a mechanism involving the exchange of filtered cations for hydrogen ions secreted by the cells of the renal tubules. These hydrogen ions are believed to be derived from the intracellular hydration of carbon dioxide under the catalytic influence of carbonic anhydrase (Pitts & Alexander, 1945). Many workers have recently tried to determine whether all the filtered bicarbonate is reabsorbed in this way or whether other mechanisms are also involved.

Pitts & Lotspeich (1946) found that very large doses of sulphanilamide, at that time the strongest carbonic anhydrase inhibitor available, reduced tubular reabsorption of bicarbonate by only one-fifth. They concluded that the ion exchange mechanism is responsible for the reabsorption of about one-fifth of the total filtered load of bicarbonate. Pitts, Ayer & Schiess (1949) and Hilton, Capeci, Kiss, Kruesi, Glaviano & Wégria (1956) have shown that excretion of chloride and bicarbonate are to some degree interdependent, suggesting that there is competition between these ions for reabsorption by some process which is not dependent on hydrogen ion secretion.

The use of acetazolamide (2-acetylamino-1:3:4-thiadiazole-5-sulphonamide), a more powerful carbonic anhydrase inhibitor synthesized by Miller, Dessert & Roblin (1950), has produced excretion of a greater proportion of the filtered bicarbonate in the urine. With moderate doses of the drug Berliner (1952) obtained excretion of about 50% of the filtered bicarbonate in the urine of dogs and Schwartz & Relman (1954) using larger doses observed excretion of apparently 80–90% of the filtered bicarbonate. On these grounds these workers postulated that there is no distinction between the mechanisms by

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which the proximal and distal portions of the renal tubule reabsorb bicarbonate, and that the whole process is dependent on the secretion of hydrogen ions. Recent observations by Schwartz, Falbriard & Relman (1958) on the relation between dose of inhibitor and plasma bicarbonate concentrations have given some indirect support to this hypothesis.

The aim of the present work was to study the relation between the dose of acetazolamide and the rate of renal reabsorption of bicarbonate, and to attempt to infer from this relation whether or not a single mechanism dependent on carbonic anhydrase could account for the findings.

METHODS

Observations on human beings. In four short-term experiments normal adult volunteers (subjects *A*, *B*, *C* and *E*) were given increasingly large quantities of sodium acetazolamide by a series of rapid intravenous injections spaced at intervals of 20 min. The first dose was 3 mg and a cumulative total of approx. 1 g (approx. 15 mg/kg body weight) was given in a period of 2 hr. A typical dose scheme was 3, 30, 70, 100, 300, 500 mg, making a total of 1003 mg.

Simultaneously measurements of glomerular filtration rate (G.F.R.) were made at intervals of 20 min using an intravenous infusion of inulin. Two control samples of blood and urine were obtained at intervals of 20 min. Thereafter blood samples were taken 1 min before each injection of acetazolamide and urine collections were accurately timed to cover the intervals between blood samples. The pH and the bicarbonate concentrations of each specimen of plasma and urine were measured. The inulin clearance was measured over two or three consecutive 20 min periods in each subject.

In one normal person (subject *D*) doses of sodium acetazolamide ranging from 1 to 1000 mg were injected intravenously at intervals of a week. The effect of each dose on the rate of bicarbonate excretion was measured by analysis of control specimens of urine and also of three specimens passed at intervals of 13 min immediately after the injection. Each experiment was done at the same time of day and the whole procedure was rigidly standardized. The glomerular filtration rate was measured each time.

In all these observations samples of arterialized blood were obtained by immersing the arm in water at 118–122° F (47·8–50·0° C) for 5–10 min and then taking specimens from a superficial forearm vein through wide-bore needles without compression of the limb. The syringes were lightly oiled and heparin was used as an anticoagulant. The subjects lay recumbent throughout the procedure except when voiding urine, which was done without catheterization. Urine was collected into vessels containing a half-inch layer of liquid paraffin.

Observation on dogs. These were made on nine mongrel dogs weighing from 10 to 14·5 kg. The animals were anaesthetized by intravenous injection of pentobarbitone sodium B.P. and anaesthesia was maintained by repeated injections of this drug.

The general procedure was similar to the short-term human experiments, except that very much larger doses (up to 1000 mg/kg) were administered. Specimens of arterial blood were taken from the first six dogs at intervals of 20 min immediately before each injection of sodium acetazolamide. In the last three experiments, samples of blood were obtained at intervals of about 1, 2, 3, 5, 7, 10 and 15 min after injection of large doses of the drug. Arterial blood was taken from polythene tubing tied in the femoral artery. Urine was collected either through a catheter tied in the fundus of the bladder or from ureteric catheters. Blood pressure was recorded continuously from either the femoral or carotid artery. A priming injection of inulin was given intravenously and a constant infusion of inulin was maintained throughout the experiment. The sodium acetazolamide was administered into the jugular vein in doses of up to 10 g. This required injection of as much as 50 ml. of fluid and occupied 1–2 min.

Analytical. Plasma for carbon dioxide (CO_2) estimation was obtained by anaerobic centrifugation in the original sampling syringes adapted by the method of Gabardi & Davenport (1949).

The total CO_2 content of both plasma and urine was measured by the manometric technique of van Slyke & Neill (1924). Blood pH was measured within a few minutes of collection by means of a Cambridge glass electrode, a Stadie anaerobic electrode chamber jacketed by water at room temperature and a Marconi battery-operated pH meter type T511D. The instrument was calibrated with standard buffer solutions. The values were corrected to 38° C by subtracting 0·014 × (38 minus room temperature) from the observed value. Urine pH was measured in the same way without correction for temperature. Plasma and urine inulin concentrations were measured by the colorimetric method of Dick & Davies (1949).

Calculations. The CO_2 tension (pCO_2) of plasma was calculated from the measured plasma pH and CO_2 content, using the Henderson–Hasselbalch equation. In accordance with current practice the rate of bicarbonate reabsorption by the tubules has been expressed as the quantity of bicarbonate reabsorbed from a litre of the glomerular filtrate. The formula employed with correction for Donnan equilibrium was:

$$\frac{(1{\cdot}05 \times \text{Mean plasma bicarbonate concentration during period or urine collection} \times \text{G.F.R.}) - (\text{Rate of bicarbonate excretion in urine})}{\text{G.F.R.}}$$

The method of calculation of the mean plasma bicarbonate concentration is of crucial importance in interpretation of the results after large doses of acetazolamide (see p. 282). In the human experiments and in the first six experiments on dogs, the plasma concentration was taken as the arithmetic mean of the samples taken immediately before and 20 min after injection of the drug. In the last three animal experiments we attempted to obtain a more accurate value of the mean plasma bicarbonate concentration by planimetric analysis of a graph relating its concentration in frequent blood samples with time.

RESULTS

Human experiments

There was in each case a systematic relation between the dose of acetazolamide administered and the rate of renal bicarbonate reabsorption, with very little variation between different persons (Table 1 and Fig. 1). As little as 3 mg (about 0·00017 m-mole/kg body wt.) of the sodium salt caused an appreciable reduction of renal bicarbonate reabsorption, a large effect was obtained from 300 mg and only a trivial further effect from 1000 mg. The general form of the curves of Fig. 1 suggests that as the dose of acetazolamide was increased bicarbonate reabsorption approached asymptotically to a limiting value equal to roughly three-quarters of the initial level, or, in other words, the final reduction of reabsorption was about 25%.

In each of the short-term experiments the plasma bicarbonate concentration showed a slight progressive fall, due, presumably, to excretion of bicarbonate in the urine. The total bicarbonate excretion in the 3 hr period of these experiments ranged from 66 to 101 m-equiv. The maximum variation in plasma CO_2 tension which occurred in any of the human experiments was 4 mm Hg.

Animal experiments

Fig. 2 shows the relation between the rate of renal bicarbonate reabsorption and the amount of acetazolamide administered (range and average values of six

experiments). After small doses of the drug, comparable in terms of body weight with those given to the human subjects, the change in bicarbonate reabsorption in dogs was of the same order but somewhat greater than that in man (Fig. 2*A*). The average reduction in reabsorption after a single dose of sodium acetazolamide of 0·08 m-mole/kg was 36%. However, comparison of Figs. 1 and 2*A* will show the much wider variation of the results in animals compared with those in human beings and also the erratic fluctuations in renal bicarbonate reabsorption, possibly related to the variation of arterial pCO_2 which occurred during the course of the animal experiments. The effects of

TABLE 1. Effect of intravenous sodium acetazolamide on bicarbonate reabsorption

Subject *B*, weight 85·5 kg

Time (min)	Sodium acetazolamide cumulative dose (mg)	Glomerular filtration rate (ml./min)	Plasma HCO_3 (m-equiv/l.)	Mean plasma HCO_3 (m-equiv/l.)	HCO_3 filtered (m-equiv/min)	HCO_3 excreted (m-equiv/min)	HCO_3 reabsorbed (m-equiv/min)	$\frac{HCO_3 \text{ reabsorbed}}{HCO_3 \text{ filtered}} \times 100$
−24½	—	—	—	—	—	0·05	4·57	98·9
	—	—	—	24·9	4·62	—	—	— 98·3
−13	—	—	—	—	—	0·11	4·51	97·7
0	3	—	24·9	—	—	0·25	4·46	94·7
+11	—	—	—	25·3	4·71	—	—	— 93·8
+25½	33	—	25·6	—	—	0·34	4·37	92·9
+36	—	174	—	—	—	0·64	4·03	86·4
	—	—	—	25·1	4·67	—	—	— 86·2
+46	103	—	24·5	—	—	0·65	4·02	86·0
+56½	—	173	—	—	—	0·73	3·77	83·9
	—	—	—	24·2	4·50	—	—	— 84·5
+76	203	—	23·8	—	—	0·67	3·83	85·0
+90	—	185	—	—	—	0·71	3·66	83·8
	—	—	—	23·5	4·37	—	—	— 84·8
+102	378	—	23·2	—	—	0·63	3·74	85·8
+112	—	—	—	—	—	0·66	3·62	84·3
	—	—	23·0	—	4·28	—	—	— 84·5
+123½	678	—	—	22·7	—	0·65	3·63	84·7
+134	—	—	—	—	—	0·66	3·55	84·5
	—	—	22·7	—	4·21	—	—	— 85·1
+148½	1003	—	22·6	—	—	0·61	3·60	85·7
+158½	—	—	—	—	—	0·63	3·57	84·8
+169½	—	—	—	22·6	4·20	0·55	3·65	86·9 — 85·9

administration of very large doses −2·5–3·5 m-mole (625–875 mg)/kg body wt.—were also variable: bicarbonate reabsorption fell by 32% in dog 2, by 67% in dog 1 and by 76% in dog 6 (Fig. 2*B*).

It should be emphasized at this point that in calculating the rate of bicarbonate reabsorption in these six experiments the 'mean' plasma bicarbonate concentration was taken as the arithmetic mean of the values immediately before and 20 min after injection of acetazolamide. Three subsequent experiments, however, indicated that large doses of sodium acetazolamide produced an abrupt but transient metabolic alkalosis which greatly influenced the true mean plasma bicarbonate in the 20 min period following injection of the inhibitor. The data from three experiments (Fig. 3) show that for each millimole (257 mg) of sodium acetazolamide injected per kilogram of body weight,

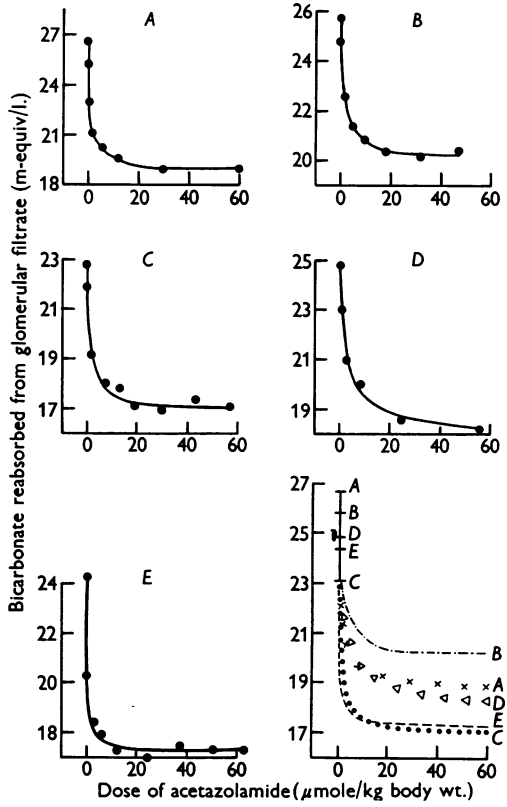

Fig. 1. Effect of increasing doses of sodium acetazolamide on tubular reabsorption of bicarbonate in five normal subjects. Dots represent experimentally determined data; continuous lines are derived mathematically (see Discussion).

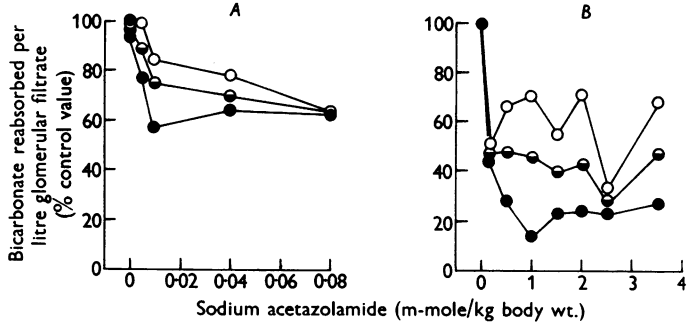

Fig. 2. Apparent effect of increasing doses of sodium acetazolamide on renal reabsorption of bicarbonate in six dogs. Results obtained when plasma bicarbonate concentration is measured at intervals of 20 min. ○, highest observed values; ●, lowest observed values; ◒, mean values in 6 dogs.

the plasma bicarbonate concentration rose within 1–2 min after injection to a peak at which it was 1·9–2·8 m-equiv/l. higher than the control level, while the blood pH increased by 0·08–0·12 units. The increments of plasma bicarbonate and pH were approximately halved by the end of each 10 min interval after injection of the drug.

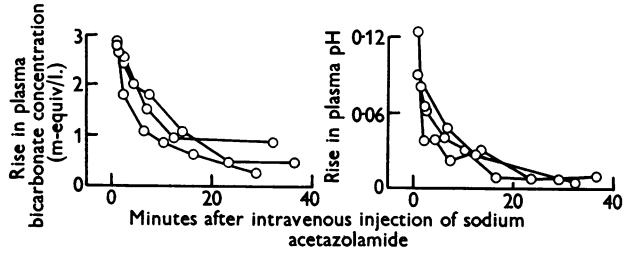

Fig. 3. Changes in plasma pH and bicarbonate concentration in dogs after injection of 1 m-mole/kg sodium acetazolamide.

Table 2 shows details of one of the experiments in which an attempt was made to allow for these immediate acid–base effects of sodium acetazolamide by collecting urine over intervals of only 10 min, while making frequent measurements of the plasma bicarbonate concentration. In the three dogs in which true mean values of plasma bicarbonate were obtained by planimetric analysis of the concentration–time curve derived from frequent sampling, the amount of bicarbonate appearing in the urine rose to 55, 60 and 52% of the filtered load. Had the results been calculated from the arithmetic mean of the first and last plasma bicarbonate levels, as was done in the earlier experiments, these values would have appeared to be 66, 78 and 74% (Fig. 4).

TABLE 2. Effect of intravenous sodium acetazolamide on bicarbonate reabsorption. Dog 7, female, weight 11 kg; anaesthesia induced with ether followed by intravenous pentobarbitone; spontaneous respiration to 90 min, respiration then controlled by pump

Time (min)	Sodium acetozolamide (g)	Glomerular filtration rate (ml./min)	Plasma pH	Plasma pCO_2 (mm Hg)	Plasma HCO_3 (m-equiv/l.)	Mean HCO_3 (m-equiv/l.)	HCO_3 filtered (m-equiv/min)	HCO_3 excreted (m-equiv/min)	HCO_3 reabsorbed (m-equiv/min)	$\frac{HCO_3 \text{ reabsorbed}}{HCO_3 \text{ filtered}} \times 100$
−48	—	—	7·28	35	15·9					
	—	52	—	—	—	15·7	0·858	0·038	0·820	95·5
−25	—	—	7·27	35	15·5					
	—	48	—	—	—	15·6	0·787	0·035	0·752	95·6
0	0·15	—	7·30	33	15·6					
	—	33	—	—	—	15·6	0·541	0·199	0·342	63·2
+34	—	—	7·26	36	15·5					
	—	30	—	—	—	15·0	0·474	0·199	0·275	58·0
+80	2·0	—	7·15	43	14·4					
+82½	—	—	7·24	41	17·0					
+89½	—	26	7·15	46	15·5	15·8	0·437	0·224	0·213	48·7
+93½	—	—	7·12	48	15·2					
	—	26	—	—	—	14·8	0·403	0·190	0·213	52·9
+120½	7·0	—	7·15	43	14·3					
+123½	—	—	7·38	38	21·5					
+125	—	47	7·31	43	20·5	20·1	0·992	0·472	0·520	52·4
+129½	—	—	7·27	41	18·1					
	—	16	—	—	—	17·4	0·293	0·161	0·132	45·1
+139½	—	—	7·22	42	16·7					
	—	25	—	—	—	16·6	0·435	0·234	0·201	46·2
+155	—	—	7·14	50	16·5					
	—	19	—	—	—	15·9	0·317	0·142	0·175	55·2
+169	—	—	7·18	42	15·3					
	—	29	—	—	—	15·3	0·467	0·166	0·301	64·5
+188	—	—	7·06	56	15·3					

We also observed a reduction in arterial pCO_2 ranging from 10 to 40% of the control level during the first minute after injection of large amounts of sodium acetazolamide. This was presumably due to direct combination of the hydrolysed alkaline salt with dissolved CO_2 in the blood, since respiration was, at the same time, often greatly depressed. The maximum change which occurred in a single animal was 51 mm Hg. The variation in pCO_2 was not less than 8 mm Hg during any experiment. This occurred despite all efforts to maintain an even plane of anaesthesia and, in some later experiments, to keep the blood pCO_2 constant by controlling ventilation with a respiratory pump.

A further complication was introduced by the rapid fall in blood pressure which followed a large dose of sodium acetazolamide. This probably produced a considerable reduction in the glomerular filtration rate, but the changes were too rapid to measure.

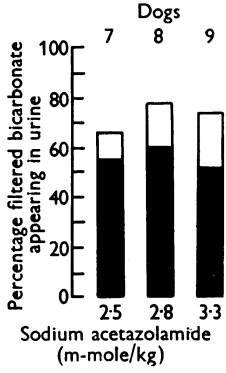

Fig. 4

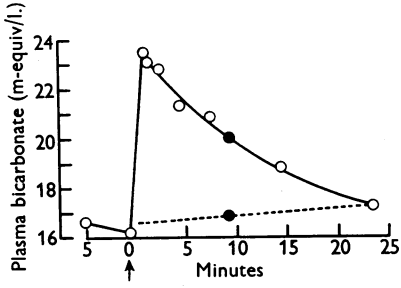

Fig. 5

Fig. 4. Effect of method of calculation on apparent rate of bicarbonate reabsorption in dogs. □, calculated from apparent plasma bicarbonate concentration; ■, calculated from true mean plasma bicarbonate concentration.

Fig. 5. Error introduced by method of calculation of mean plasma bicarbonate. Dog, 12 kg, received 8 g sodium acetazolamide intravenously at arrow ↑.

DISCUSSION

The aim of this work was to see whether the relation between increasing doses of acetazolamide and the rate of renal reabsorption of bicarbonate was consistent with the hypothesis that reabsorption is accomplished by a single mechanism requiring carbonic anhydrase. If inferences about this are to be drawn from such a dose–response relation it is manifestly essential to know either that the effects of sodium acetazolamide on bicarbonate reabsorption are entirely due to its inhibitory action on carbonic anhydrase, or that allowance can be made with sufficient accuracy for any other actions of the drug. It is clear that when the alkaline sodium salt of acetazolamide is used, the effects

of the drug are, in fact, not confined to inhibition of carbonic anhydrase, since large amounts injected intravenously cause a metabolic alkalosis, with a simultaneous reduction of arterial pCO_2. An obvious explanation of this, already suggested by Maren (1956), is that the alkaline salt reacts with carbonic acid to form sodium bicarbonate in the plasma. A detailed study of this reaction is to be presented in a further paper; but in the present context the important point is that the bicarbonate released into the plasma will automatically increase the filtered load of bicarbonate presented to the tubules for reabsorption. However, this change is transient and may not be apparent if the blood is not analysed often enough. The significance of this is illustrated in Fig. 5. The mean plasma bicarbonate concentration in the 23 min period of urine collection after injection of the drug would be 16·9 m-equiv/l. if this were calculated from the average of the values immediately before injection and 23 min later, i.e. if one were ignorant of the interim changes in plasma bicarbonate concentration. The mean plasma bicarbonate concentration, however, estimated planimetrically from a graph of time against concentration, would be 20 m-equiv/l. Taking this as the true mean plasma concentration, the calculated rate of renal bicarbonate reabsorption during the period of urine collection would be 3·1 m-equiv/l. of glomerular filtrate higher than that given by the simple two-value calculation. In other words, taking only two samples for bicarbonate analysis, one immediately before and one 23 min after injection of the drug, would underestimate reabsorption by about 16%. Non-recognition of such transient changes in plasma bicarbonate concentration may have accounted, to some extent at least, for the apparent almost total abolition of bicarbonate reabsorption reported by Schwartz & Relman (1954) in response to injections in dogs of sodium acetazolamide 500 mg/kg body wt.; no experimental details of this work are available, however.

In the ideal experiment for our purpose, only one variable, the dose of acetazolamide, would be deliberately altered, and other factors which are known to influence bicarbonate reabsorption, the plasma bicarbonate concentration, arterial pCO_2 and G.F.R., would remain constant. In the observations on normal persons described above there was a reasonably close approach to this. But in the animal experiments, particularly those involving large doses of the inhibitor, there were changes in arterial pCO_2 and almost certainly in the G.F.R. which introduced imponderable errors in calculation and interpretation of the results of the animal experiments. This, coupled with the error which would be involved in attempting to allow for the alkalizing effect of the drug, makes invidious any attempt to draw firm conclusions from the high-dose experiments on dogs reported here.

As might be expected, the magnitude of the metabolic alkalosis induced by the drug's alkalizing effect appears to be proportional to the dose given. Applying the data from animal experiments to man it is calculated that

administration of 1000 mg to an adult of 50 kg body weight would cause a peak increase of only about 0·20 m-equiv/l. in plasma bicarbonate. This would represent a 1% increase in plasma containing 20 m-equiv/l. a change which would be within the limits of error of measurement. Thus, in the observations on human beings, in whom the maximum dose administered was only 16 mg/kg body wt., the alkalosis produced by the drug can safely be regarded as negligibly small.

Significance of the experimental results in human beings

The essence of the findings here is that as the dose of the inhibitor is increased bicarbonate reabsorption by the renal tubules diminishes in a characteristic way and appears to approach asymptotically a limiting value equal to roughly 75% of the initial level. The inference which might be drawn is that the drug has inhibited one mechanism of tubular bicarbonate reabsorption which is dependent on the enzyme, the reabsorptive capacity of which is only about 25% of the normal load of bicarbonate presented to the tubules. The validity of this inference depends on whether the rate of bicarbonate reabsorption does genuinely approach a limiting value or not. The evidence would be strengthened by extending tenfold the range of dosage beyond 1000 mg, but this was not considered to be a justifiable hazard for our volunteers, and would, in any case, have involved large corrections for the alkalizing effect of the drug.

There is good evidence (Davenport, 1945) that the reaction between the sulphonamide inhibitors of carbonic anhydrase and the enzyme is monomolecular. This reaction, by the law of mass action, can therefore be written as follows:

$$\frac{(E)\,(A-EA)}{(EA)}=K, \tag{1}$$

where E is the concentration of free enzyme at equilibrium,
A is the original concentration of inhibitor,
EA is the concentration of enzyme + inhibitor complex at equilibrium, and
K is the equilibrium constant.

In the following argument we attempt to demonstrate that infinite doses of the inhibitor would not produce an appreciably greater reduction in the amount of bicarbonate reabsorbed by the tubules than was produced by the largest doses used in our observations on human subjects.

The following assumptions are made:

(1) (EA) is negligibly small in comparison with (A).

(2) The concentration of inhibitor in the renal tubule cells is directly proportional to the cumulative dose of acetazolamide administered.

(3) The amount of bicarbonate reabsorbed by the renal carbonic anhydrase mechanism is proportional to the concentration of active enzyme in the tubule cells.

No correction has been made for the excretion of drug during the experimental period.

Equation (1) may then be modified to:

$$\frac{(R-X)(D)}{(C-R)}=K', \tag{2}$$

where R is the observed amount of bicarbonate reabsorbed from 1 l. of glomerular filtrate after a dose D of acetazolamide,

C is the amount of bicarbonate reabsorbed before administration of the drug, and

X is the amount of bicarbonate which would be reabsorbed from each litre of glomerular filtrate if carbonic anhydrase activity were nil.

Then $(C-R)$ is the reduction in bicarbonate reabsorption due to inhibition of enzyme and $(R-X)$ is the amount of bicarbonate being reabsorbed by the uninhibited enzyme.

TABLE 3. Values of constants obtained by substituting results observed for different subjects in equation (2)

Subject	X	K'	C
A	18·9 (0·11)	15·2 (0·59)	26·5
B	20·1 (0·14)	31·1 (5·51)	25·5
C	17·0 (0·12)	22·6 (3·94)	22·8
E	17·2 (0·11)	8·2 (1·24)	24·3
D	18·2 (0·21)	38·0 (6·51)	24·9

The standard errors are given within brackets: those quoted for subjects A, B and C are based on a common estimate of the root mean squared deviation of R from the curves of these subjects. Those of subjects E and D are based on a common estimate for all curves.

Equation (2) has been fitted to the results from each subject, using the method of least squares, thus providing estimates of X, K' and C and their standard errors (Table 3). The fitted relationship from the equation is illustrated by the continuous curves in Fig. 1 along with the observations for each subject. These suggest good agreement between the law and the experimental results. The standard errors of X are all small compared with the estimates of X, confirming that the lower limit approached by the curve as D increases is substantially and significantly greater than zero.

It is always possible that some other mechanism may take over as the dose of inhibitor increases beyond the range experienced and that the true relationship may diverge from the fitted one beyond this range. However, our experiments provide no evidence to suggest that large doses of acetazolamide in man would produce complete excretion of the bicarbonate filtered by the glomeruli.

We have also been unable to confirm the observation that in dogs 90% of filtered bicarbonate is excreted in the urine after injection of 500 mg/kg body wt. of acetazolamide. We consider, therefore, on the basis of these results that there are at least two mechanisms by which tubular reabsorption of bicarbonate occurs and that only one is dependent upon carbonic anhydrase.

SUMMARY

1. The effect on the renal excretion of bicarbonate of intravenous injection of increasing doses of sodium acetazolamide up to a total of 1 g has been studied in five normal persons.

2. The maximum amount of bicarbonate excreted was 21–29% of the load filtered by the glomeruli.

3. Similar experiments were carried out on nine dogs, each of which received over 500 mg/kg body weight of sodium acetazolamide.

4. The large doses of sodium acetazolamide given to dogs produced a considerable but transient increase in plasma bicarbonate concentration.

5. When due allowance was made for the increased filtered load of bicarbonate produced by the sodium acetazolamide, the maximum amount of bicarbonate excreted in the urine of the dogs after large doses of the drug was 60% of the filtered load.

6. A theoretical analysis of our results supports our conclusion that in man the renal tubules reabsorb about 75% of the filtered bicarbonate by one or more mechanisms which are not dependent on carbonic anhydrase.

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