SELECTIVE RENAL CARBONIC ANHYDRASE INHIBITION WITHOUT RESPIRATORY EFFECT: PHARMACOLOGY OF 2-BENZENESULFONAMIDO-1,3,4-THIADIAZOLE-5-SULFONAMIDE (CL 11,366)[1, 2]

DAVID M. TRAVIS, CHRISTINE WILEY, BOHDAN R. NECHAY AND THOMAS H. MAREN

Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, Florida

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Effective doses of most inhibitors of carbonic anhydrase usually elicit physiological responses at various transport and secretory sites without discrimination. Acetazolamide, for example, produces full effects in kidney, eye, stomach, pancreas and red cell at the very same dose (Maren, 1963b). Multiple responses occurring simultaneously serve to complicate study of any single system.

Inhibition of erythrocytic enzyme in particular has complicated the use of acetazolamide in the study of effects of inhibition at other sites. Carbonic anhydrase in red cells catalyzes the CO_2–carbonic acid reaction which is important for CO_2 exchange and HCO_3^- transport at peripheral and pulmonary capillary beds (Berliner and Orloff, 1956). Thus red cell carbonic anhydrase inhibition leads to a rise in CO_2 tension in the tissues (Mithoefer and Davis, 1958) and a fall in CO_2 tension in the alveoli of the lungs (Tomashefski *et al.*, 1954). An increase in pulmonary ventilation usually minimizes the ensuing CO_2 retention. However, net shifts in acid-base equilibrium may elicit physiological responses or alter the response to inhibitor in certain tissues, including kidney (Maren, 1956), ciliary structures (Wistrand and Maren, 1960; Wistrand *et al.*, 1961a), cerebral blood vessels (Wistrand *et al.*, 1961a) and stomach (Byers *et al.*, 1962).

The achievement of organ specific carbonic anhydrase inhibition would then offer some distinct experimental and therapeutic advantages. For instance, isolation of the renal effect would provide the means of producing cation and HCO_3^- loss unmixed with acute CO_2 retention. Also, in patients with respiratory failure and hypercapnia this renal response could be obtained without the risk (Platts and Hanley, 1956) of immediate further impairment of the ability to excrete CO_2 through the lungs.

Three observations suggested that separation of renal from erythrocytic and other effects should be possible: 1) a compound related to acetazolamide (fig. 1), 2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide (CL 11,366) synthesized by Vaughan *et al.* (1956), diffuses from plasma into red cells less readily than other inhibitors (Maren *et al.*, 1961); 2) this compound is secreted by renal tubules and hence concentrates in the kidney (Maren, 1963b); and 3) the concentration of carbonic anhydrase in red cells is at least 3-fold that found in other tissues, including kidney (Maren, 1962). By appropriate choice of dose of this powerful inhibitor, it is possible to separate renal from erythrocytic and other physiological responses.

This paper describes and compares the distribution and physiological effects of CL 11,366 and acetazolamide over a range of single i.v. doses and of time. In addition, concepts previously developed for the relationship between fractional enzyme inhibition in kidney and HCO_3^- loss are extended to the red cell and respiration.

METHODS. Mongrel dogs anesthetized with thiopental or pentobarbital were used in experiments for distribution of drug in kidney, brain and eye and for determining the changes of pressure in ocular and cerebrospinal fluids and the changes in gastric acid secretion after feeding. Conscious beagles were subjects for measurement of renal and respiratory function.

Animals received tap water and equal portions of nonmedicated Purina dog food and canned horse meat supplemented with vitamins A and D. Food was excluded for 24 hours before all studies and during experiments where measurements extended less than 12 hours.

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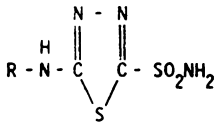


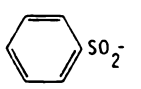
	CL 11,366	ACETAZOLAMIDE
R-		CH_3CO -
K_1 (37^0)	5×10^{-9} M	60×10^{-9} M
Ether Partition	0.001	0.14
PKa	3.2	7.4
Red Cell Diffusion	Low	Moderate
Plasma Bound	93%	50%

FIG. 1. Structural formulas and properties of CL 11,366 and acetazolamide.

Procedure for renal and respiratory measurements. Female beagles, trained to breathe into a mask without restraint, were studied singly in a quiet room. Temperature of the room was 22 to 25°C and varied less than 2° during a period of 6 hours.

Initially the dogs received tap water (20 ml/kg) by stomach tube. Thirty minutes later the bladder was drained by catheter. Urine was then collected at 30-minute intervals into graduated cylinders under toluene, followed by two 10-ml washes of the bladder with sterile distilled water. At the midpoint of urine collection periods blood was taken from a leg vein, with as little stasis as possible, into syringes with dead space containing heparin (200 units per ml) in 0.9% NaCl solution. Respiratory measurements were taken at the time of blood sampling. These included sampling of end-tidal expiratory gas for estimate of alveolar CO_2 tension during a period of 8 minutes, including a 4-minute collection of expired gas by the techniques described below.

After two control urine collections, drug was injected i.v. over a period of 1 minute. Required amounts of drug were dissolved in 1 to 1.6 moles of NaOH (as 1 N solution) per mole of drug and diluted 10-fold with 0.9% NaCl or, in the case of doses above 10 mg/kg, with water. The collection of urine, blood and gas samples continued for several hours after low doses and for many hours after high doses.

Respiratory measurements. Conscious beagles sat and breathed comfortably into a mask held snugly over the face. Noise and physical disturbance were kept to a minimum so that a constant breathing pattern was obtained. Occasional rise of room temperature led to panting respiration which altered results, and these are not included in the figures or tables.

The face mask consisted of a rubber glove from which four fingers were cut and tied and one finger was cut and fitted over the open neck of the top quarter of a plastic bottle. This plastic top inside the end of the glove prevented collapse of the rubber against the nose and helped maintain a more constant dead space within the mask. The hand of the glove fitted well over the face and mouth. The mask was attached by the bottle neck to a respiratory valve made of thick lucite and equipped with "J" valves. Dead space of the valve was 60 ml and that of the mask and connecting tube about 20 ml. The valve had sampling ports for both inspiratory and expiratory chambers for continuous sampling of gas into a rapid infrared CO_2 analyzer at a rate of 400 ml per minute.

End-expiratory "alveolar" CO_2 measurements were taken from the inspiratory chamber as close as possible to the nose. This technique in our hands gives a close approximation to true alveolar CO_2 tension (P_{ACO_2}) as measured by close agreement with arterial CO_2 tension (Julian *et al.*, 1960). Expired gas was collected into a Douglas bag and volume measured in a spirometer. Ventilation, alveolar ventilation and respiratory gas exchange were obtained by methods and calculations previously used (Travis *et al.*, 1960).

Samples of expired gas were collected and stored in gas tight syringes coated with lithium chloride paste and fitted with stopcocks. Any leaks were detected by compressing the volume to half while the tip was held under water. Syringes were stored upright with the weight of the syringe on the plunger.

Determination of pressures in cerebrospinal fluid (CSF) and eye and of gastric acid secretion. The methods described by Wistrand *et al.* (1961a,b) were used to study responses of intraocular and CSF pressures. The methods of Byers *et al.* (1962) were employed to study postfeeding gastric acid secretion.

Analytical techniques. Whole blood pH was measured immediately after collection at 37°C (Cambridge Model R, glass microelectrode assembly) and urine pH at room temperature (Photovolt meter, Model 115) with 0.014 pH unit subtracted for each degree below 37°C. Blood samples were centrifuged and plasma and red cells separated for analysis. Total CO_2 content of plasma and urine was determined in 0.03 ml of sample in the manometric microgasometer (Knights *et al.*, 1957). Drug concentrations were measured by the enzymic method (Maren *et al.*,

1954b). For CL 11,366 two minutes of equilibration of drug with enzyme were allowed before addition of buffer in many of the tests since this is preferable to the nonequilibrated method. Cl^- was measured by Brun's modification of the Schales and Schales method (Smith, 1956), and Na^+ and K^+ by Baird Atomic flame photometer, lithium internal standard method. Samples of expired gas were analyzed for CO_2 and O_2 by the volumetric method of Scholander (1947). Rapid analysis of expiratory gas for CO_2 was performed using the Beckman infrared Model LB 1.

Calculations and comparisons. Volumes of ventilation were expressed in BTPS (37°C, ambient pressure, saturated with water vapor) and values for CO_2 and O_2 exchange in STPD (0°C, 760 mm Hg pressure, dry). Alveolar ventilation was obtained from the Bohr formulation using the fractions of CO_2 in expired and end-tidal "alveolar" gas. Blood CO_2 tension was derived from the pH of whole blood and the CO_2 content of true plasma by the line chart of Van Slyke and Sendroy (1928). Total urinary CO_2 output was expressed as HCO_3^- excretion in tables and figures.

Fractional inhibition of enzyme (i) was obtained in the manner described by Maren (1963b) from total inhibitor concentration (I_0) using the relation

$$I_0 = K_I\left(\frac{i}{1-i}\right) + iE_0 \quad \text{(Equation 1)}$$

derived from the equilibrium of inhibitor with enzyme, where K_I is the dissociation constant at 37°C and E_0 is total enzyme concentration for the tissue in the untreated animal. Calculations of i shown in the tables were made using E_0 of 30 μmol per liter for red cells (Maren, 1962) and 10 μmol per kg ± 0.2 S.E. (n = 14) for renal cortex; E_0 for medulla of kidney for this calculation was 1 μmol per kg ± 0.3 S.E. (n = 11).

An additional calculation of i was made in table 3 using the relationship

$$i = \frac{I_{free}}{I_{free} + K_I} \quad \text{(Equation 2)}$$

That portion of total inhibitor in red cells which can be washed out in three washes (the diffusible component, RBC_d) is substituted for I_{free} for comparison of the result with i as calculated from I_0 (Maren *et al.*, 1961; Maren, 1963b).

Molecular weights are 330 for CL 11,366 and 222 for acetazolamide.

Comparisons with acetazolamide refer to Maren *et al.* (1954a) unless otherwise stated. Figure 1 summarizes data from previous work (Maren *et al.*, 1961; Maren, 1963a,b; Wistrand *et al.*, 1961b) giving K_I, pKa, red cell diffusion, plasma binding and the partition of inhibitor between ethyl ether and phosphate buffered saline of pH 7.4.

RESULTS. *Distribution.* CL 11,366 provides a contrast to acetazolamide in its different distribution in kidney, plasma, erythrocytes, eye, CSF and aqueous humor.

Kidney: Table 1 shows that CL 11,366 over a large range of dose gives an I_0 of renal cortex 3- to 9-fold that of plasma, whereas acetazolamide produces an I_0 in renal cortex less than 2-fold the plasma inhibitor. The higher accumulation of CL 11,366 in kidney was related to its more rapid appearance in the urine. About 60% of the dose was recovered in 1 hour (fig. 2) compared to 20% of acetazolamide in a similar period.

Plasma: Rapid excretion of CL 11,366 leads to plasma decay with a half-life of about 20 minutes (fig. 2), much shorter than the 100 minutes for acetazolamide. When the concentration of CL 11,366 fell below 1 μg per ml, the rate of decay changed, as previously calculated for acetazolamide (Maren, 1962).

Erythrocytes: Though more highly concentrated in kidney, CL 11,366 enters red cells less readily than acetazolamide at equivalent plasma concentrations *in vivo* (tables 1 and 3) and *in vitro* (Maren *et al.*, 1961). Additional data obtained in this laboratory (L. Holder, personal communication) show that the rate for entry of acetazolamide from dog plasma into red cells *in vitro* was ten times that of CL 11,366. It was of interest that this difference was essentially obliterated when the diffusion from saline into red cells was measured. This suggests that plasma binding (fig. 1) plays the dominant role in the difference in red cell diffusion between the two inhibitors (Maren *et al.*, 1961).

Figure 3 indicates that decay of CL 11,366 from erythrocytes occurred in two phases after saturation of receptors: the half-life of the first slope was 12 hours and that of the second about 4 days, similar to acetazolamide (Maren *et al.*, 1961; Maren, 1962). These two slopes are regarded as dissociation of inhibitor first from nonenzymic receptor and then from carbonic anhydrase itself. However, similarity of the actual values for these half-lives of the two drugs may be fortuitous due to a combination of

TABLE 1

Separation of renal from respiratory responses to carbonic anhydrase inhibition by CL 11,366 compared with acetazolamide[a]

Dose	I_0 at 15 min[b]				i[c]		Physiological Responses		
	Plasma	RBC	Renal cortex	Renal medulla	RBC	Renal cortex	Mean fall $P_{A_{CO_2}}$[d]	Peak rise CSF pressure[e]	Urinary HCO_3^- 30 min
mg/kg	*μM*	*μM*	*μmol/kg*	*μmol/kg*			*%*	*%*	*μEq/min*
CL 11,366 i.v.									
0.1	—	—	8	1	—	0.8	—	—	42 ± 4(6)
0.3	2 (3)	3 (3)	18 (3)	9 (3)	0.13	0.9994	—	—	73 ± 8(7)
1	6 (3)	6 (3)	21 (3)	15 (3)	0.20	0.9995	0 (2)	0	93 ± 8(6)
3	23 (3)	28 (3)	97 (3)	128 (3)	0.93	0.99994	8 (5)	0 (2)	109 ± 13(4)
10	109 (3)	73 (3)	250 (3)	390 (3)	0.9999	0.99998	32 (3)	110 (2)	94 ± 9(5)
Acetazolamide i.v.									
1	2	15	—	—	0.5	—	0 (3)	0	33 ± 8(8)
2	9	32	—	—	0.97	—	2 (3)	—	72 ± 9(9)
3	12	32	—	—	0.97	—	—	0 (2)	—
5	40	54	75 ± 8(5)	34 ± 4(4)	0.998	0.999	34 (3)	100 (3)	88 ± 5(13)
10	—	—	—	—	—	—	28	32	118 ± 10(9)
20	165	133	207 ± 7(4)	95 ± 8(7)	0.9994	0.9997	33 (2)	—	130 ± 12(7)

[a] Means ± S.E. are given. Number of experiments in different animals is one except as noted in parentheses. Values of I_0 and urinary HCO_3^- are in part from Maren (1963b).

[b] Inhibitor concentrations are shown for unwashed red cells and perfused kidney. Time refers to minutes after drug.

[c] Fractional inhibition of enzyme was calculated as described in METHODS. Since E_0 for medulla is 1 μmol per kg, only a tenth that for cortex, i for medulla (not shown) would at each dose be higher than for cortex at the I_0 values given.

[d] Alveolar CO_2 tensions at 15 and 45 minutes before and after drug were averaged separately. The mean per cent fall after drug was obtained from the differences between these averages. A fall in $P_{A_{CO_2}}$ indicates the appearance of a "blood-alveolar" gradient for CO_2 since venous CO_2 tension remained nearly constant.

[e] Peak rises in cerebrospinal fluid pressures occurred at about 15 minutes after the drug in those cases where changes developed. One animal included in the average at 5 mg/kg acetazolamide did not show a pressure response.

factors which offset each other, such as differences in K_I, plasma binding and rates of urinary excretion of inhibitors.

By contrast, the two compounds differ in their rates of decay from red cells of that portion of erythrocytic inhibitor not bound either to enzymic or to nonenzymic receptor, *i.e.*, "diffusible" inhibitor, RBC_d. Table 3 shows that RBC_d parallels the plasma decay and falls much faster with CL 11,366 than with acetazolamide.

Other tissues: Although CL 11,366 enters kidney in high concentration and red cells in moderate amount depending on the gradient from plasma, the initial distribution to other cells is probably less. At doses of 10 and 20 mg per kg body weight CL 11,366 i.v., the volumes of distribution, determined from extrapolation of plasma decay to zero time (fig. 2 and table 3), were 220 and 250 ml per kg body weight. Such values for acetazolamide are about 400 ml per kg. Exclusion of CL 11,366 from CSF and aqueous humor (less than 0.6 μM, the limit of the method) reflects high plasma binding, the poor solubility in lipid and the high degree of ionization at physiological pH. Nevertheless, CL 11,366 enters certain sites containing carbonic anhydrase. For example, I_0's in choroid plexus were 10, 33 and 67 μM (means of two experiments) 15 minutes after 1, 3 and 10 mg/kg i.v., respectively.

Renal effect of CL 11,366 and acetazolamide. CL 11,366 gives full renal responses at smaller doses than acetazolamide as shown by cation loss (Kuehn *et al.*, 1959) and HCO_3^- output

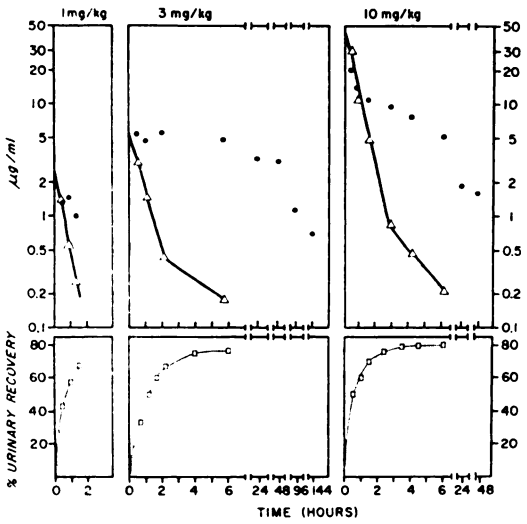


FIG. 2. Decay of CL 11,366 in erythrocytes (solid circles) and plasma (open triangles) and recovery in urine after different intravenous doses of drug at zero time.

Values shown represent one animal at each dose except for blood values in the first 6 hours which are means of experiments in two animals.

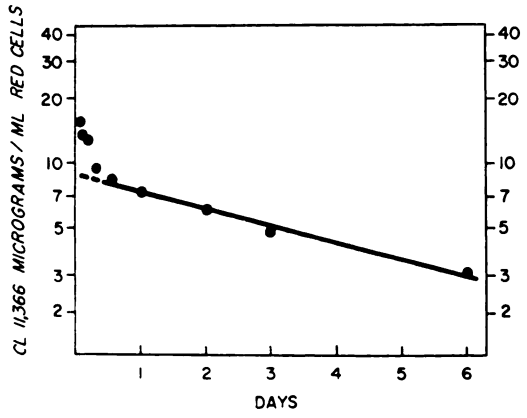


FIG. 3. Decay of inhibitor in washed erythrocytes after CL 11,366 (20 mg/kg i.v.).

Plasma decay and other data from the same experiment are given in table 3.

(Maren, 1963b). Table 1 and figures 4 and 5 illustrate this difference and permit comparison of renal and erythrocytic effects at various doses of the two inhibitors (see Respiratory effects). It is significant that CL 11,366 produced about the same initial HCO_3^- output at doses which did and doses which did not affect respiration. Table 2 indicates that duration of renal HCO_3^- excretion was also about the same at a dose (10 mg/kg) which gave acute respiratory effects and at doses (1 and 3 mg/kg) which, while giving a maximal renal effect, gave no acute respiratory effect. The duration of renal response to CL 11,366 at these doses was about 4 hours. This duration reflects the rate of decay of I_{free} from renal cortex. For example, Maren (1963b) showed that with 1 mg/kg, I_{free} is essentially zero by this time.

TABLE 2

Duration of renal response to CL 11,366[a]

Urine Collection Interval	1 mg/kg i.v.		3 mg/kg i.v.		10 mg/kg i.v.	
	HCO_3^-	pH	HCO_3^-	pH	HCO_3^-	pH
min	*μEq/min*		*μEq/min*		*μEq/min*	
−30	7	6.7	12	7.2	—	6.4
0	CL 11,366 i.v.					
+30	126	7.8	130	7.7	63	7.5
+60	111	7.9	124	8.0	97	7.7
+90	84	8.0	82	8.0	43	7.9
+150	54	8.1	55	7.9	44	7.9
+240	32	8.1	20	7.8	12	7.7
+360	8	7.3	2	6.9	—	6.7

[a] One experiment at each dose is shown. Another experiment at each dose gave similar results. Different animals were used.

Fractional inhibition of carbonic anhydrase in renal cortex was 0.999 or greater at maximal HCO_3^- excretion rates (Maren, 1963b). These values are presented in table 1 for comparison with the values for red cells and with respiratory effects.

Electrolyte excretion pattern was like that found with acetazolamide (Berliner *et al.*, 1951). Both Na^+ and K^+ were excreted along with HCO_3^-, but there was no increase in urinary Cl^- output (data not given).

Respiratory effects of carbonic anhydrase inhibition by CL 11,366 and acetazolamide. Two characteristic acute changes in respiration were used as indicators of the physiological response to erythrocytic inhibition: 1) hyperventilation and 2) fall in $P_{A_{CO_2}}$ (see DISCUSSION). Table 1 and figure 4 show that CL 11,366 produced no change in $P_{A_{CO_2}}$ 15 and 45 minutes after i.v. doses of 1 and 3 mg/kg which gave maximal renal HCO_3^- excretion, making the separation of renal from respiratory responses complete. Inspection of table 1 and figure 4 shows that a dose of 10 mg/kg (ten to twenty times that at the

TABLE 3

Erythrocytic carbonic anhydrase inhibition and respiratory response[a]

Time[b]	I_0[c]			i[d]		Blood pH	Plasma CO_2 Content	Respiration[e]	
	Plasma	RBC_0	RBC_d	RBC_0	RBC_d			$P_{A_{CO_2}}$	$\dot{V}_E$
min	*μM*	*μM*	*μM*				*mM*	*mm Hg*	*liters/min*
C	0.2	7	0	0	0	7.42	25.0	37	1.9
0	Acetazolamide 20 mg/kg (90 μmol/kg) i.v.								
5	191	165	115	0.9996	0.9995	7.47	25.1	27	2.5
30	152	115	56	0.9993	0.9989	7.35	23.1	26	3.2
180	28	70	33	0.9985	0.9982	7.32	18.4	25	3.5
360	5	42	4	0.9949	0.985	7.33	17.2	27	2.5
420	3	37	2	0.992	0.97	7.35	18.6	32	3.3
480	—	34	5	0.985	0.988	7.31	19.3	34	3.4
C	—	2	—	—	—	7.40	24.2	38	2.1
0	CL 11,366 20 mg/kg (60 μmol/kg) i.v.								
5	157	93	—	0.99993	—	—	—	32	3.4
30	66	75	21	0.99989	0.99977	7.35	23.5	27	3.0
75	25	47	2	0.99971	0.9974	7.30	22.0	24	3.7
130	9	41	—	0.99955	—	7.33	21.0	31	2.3
180	4	40	0.8	0.9995	0.9937	7.34	20.5	32	2.1
240	1	39	0.8	0.99945	0.9935	7.31	20.5	36	2.3
700	—	25	—	0.82	—	7.32	18.6	38	3.2

[a] A 9-kg beagle was studied in two experiments a week apart. An additional experiment with each drug in the same animal gave similar results.

[b] C represents control values obtained 45 and 15 minutes before drug; the mean is shown. Times in relation to drug injection refer to start of respiratory gas collection following which blood was drawn from a leg vein.

[c] I_0 = inhibitor concentration. Small amount of inhibitor in controls is presumed to be from a previous experiment. RBC_0 = I_0 in unwashed red cell. RBC_d = concentration of diffusible inhibitor in red cells = RBC_0 − (inhibitor concentration in red cells after three washes).

In the acetazolamide experiment red cells were separated from plasma in 12-ml centrifuge tubes and values for RBC_0 are given without correction of inhibitor concentration trapped in red cells containing 8% plasma. In the CL 11,366 experiment red cells were separated from plasma in hematocrit tubes in such a way that trapping was negligible.

[d] Details for calculation of fractional inhibition of enzyme (i) are given in the section on METHODS.

[e] Alveolar CO_2 tension ($P_{A_{CO_2}}$) and total volume of expired gas ($\dot{V}_E$) were measured during a period of 4 minutes.

threshold for full renal effect) was required to give a fall in $P_{A_{CO_2}}$. By contrast, doses of acetazolamide giving clearly maximal renal HCO_3^- excretion also elicited a fall in $P_{A_{CO_2}}$ (table 1 and fig. 5). The ventilatory changes shown in figure 6 parallel these changes of $P_{A_{CO_2}}$ and indicate that there is a range of dose, 0.3 to 3 mg/kg, of CL 11,366 which elicits full HCO_3^- excretion without ventilatory effect. The range of dose for such separation of responses by acetazolamide is very narrow and occurs only at submaximal renal HCO_3^- excretion.

Not only was the erythrocytic respiratory effect eliminated over a wide range of dose of CL 11,366 giving renal responses, but at doses above this range the respiratory effects were of shorter duration than those with acetazolamide. For example, 10 mg/kg i.v. CL 11,366 produced a fall in $P_{A_{CO_2}}$ and a rise in ventilation lasting less than 2 hours. An equimolar dose of acetazolamide elicited similar changes for twice as long (not shown). Another example appears in table 3 which demonstrates that a shorter duration of acute changes in ventilation after CL 11,366 reflects the faster plasma decay and decrement of RBC_d of this inhibitor compared to acetazolamide.

A fall in $P_{A_{CO_2}}$ immediately after injection of

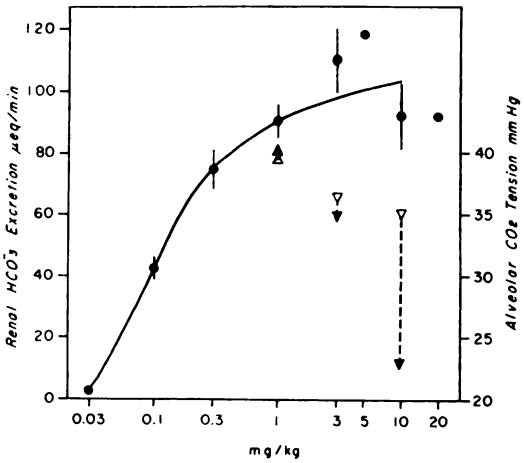


FIG. 4. Simultaneous renal and respiratory responses to CL 11,366 i.v. in conscious beagles.

Solid circles represent mean rates of urinary HCO_3^- excretion, with solid vertical lines as S.E., during 30 minutes after drug. Predrug HCO_3^- excretion was 2μ Eq/min or less in all experiments. Number of animals was 4 to 7.

Solid triangles represent alveolar CO_2 tension after drug and open triangles before drug. Magnitude of changes is indicated by dashed lines and the direction of change from control values by orientation of the triangles to form arrows. For each experiment the values at 15 and 45 minutes before and after drug were averaged separately. Shown are means for these averages in 2, 5 and 3 animals at doses of 1, 3 and 10 mg/kg, respectively. Peripheral venous CO_2 tension remained nearly constant, the mean changes being 0, −1.5 and −2 mm Hg at the three doses given. An additional experiment at 20 mg/kg is summarized in table 3.

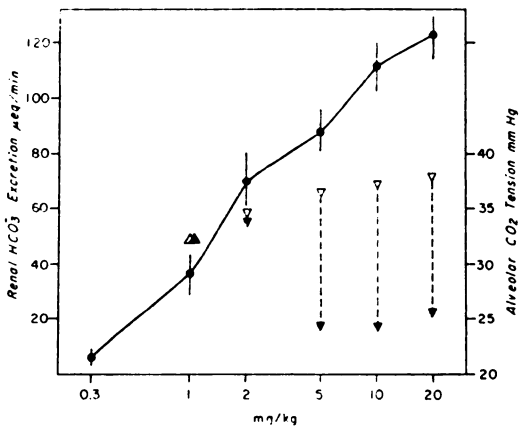


FIG. 5. Renal and respiratory responses to acetazolamide i.v. in conscious beagles.

The two parameters (symbols and conventions the same as fig. 4) were studied in separate experiments. Number of dogs was 7 to 13 for urinary HCO_3^- excretion.

Number of animals for respiratory effect was 3 except at 10 mg/kg (n = 1) and 20 mg/kg (n = 2). A fall in alveolar CO_2 tension indicates a blood-alveolar gradient, since venous CO_2 tension in these experiments changed less than 1 mm Hg.

inhibitors actually represents a widening of the normally narrow gradient of CO_2 tension between tissues and alveoli (see DISCUSSION). Effective erythrocytic inhibitory doses of the drugs produced little change in CO_2 tensions of venous (figs. 4 and 5, legends) or arterial (a fall of about 4 mm Hg; see also Wistrand *et al.*, 1961a) blood at 15 and 45 minutes in conscious dogs. Tissue CO_2 tension, not measured in the present work, is higher than arterial or venous CO_2 tension. Therefore, the magnitude of the fall in $P_{A_{CO_2}}$ represents a minimal figure for the widened gradient of CO_2 tension from tissues to alveoli created by erythrocytic inhibition.

The lack of persistent buildup of CO_2 in the blood is substantiated by the maintenance of a nearly constant ratio of CO_2 output to O_2 uptake (respiratory exchange ratio, R). At 15 and 45 minutes after effective erythrocytic inhibitory doses of the two drugs, R was within a range of ±0.07 from the mean value before drug. However, a transient fall in R was observed within 5 minutes after i.v. administration of 20 mg/kg of either inhibitor in the experiments recorded in table 3. R fell from 0.70 to 0.53 after acetazolamide and from 0.70 to 0.62 after CL 11,366. Changes of similar direction and magnitude were observed in additional experiments with each drug in the same dog. Except during this transient fall in R, the increase in alveolar ventilation is apparently sufficient to compensate for the lowered $P_{A_{CO_2}}$ so that CO_2 output is maintained.

Other responses to CL 11,366. Acetazolamide in the lowest doses which are maximally effective for both renal and red cell carbonic anhydrase (5 or 10 mg/kg) is accompanied by other responses. These include transient elevation of CSF pressure, lowering of intraocular pressure and CSF flow, and acute inhibition of postfeeding gastric acid secretion. CL 11,366 at 3 mg/kg, well above the amount required for maximal renal effect, was devoid of both erythrocytic and these other responses. These differences are documented as follows.

The immediate rise in CSF pressure following acetazolamide is regarded as a manifestation of CO_2 retention in brain tissue secondary to inhibition of red cell carbonic anhydrase (Wistrand *et al.*, 1961a). No change in CSF pressure was

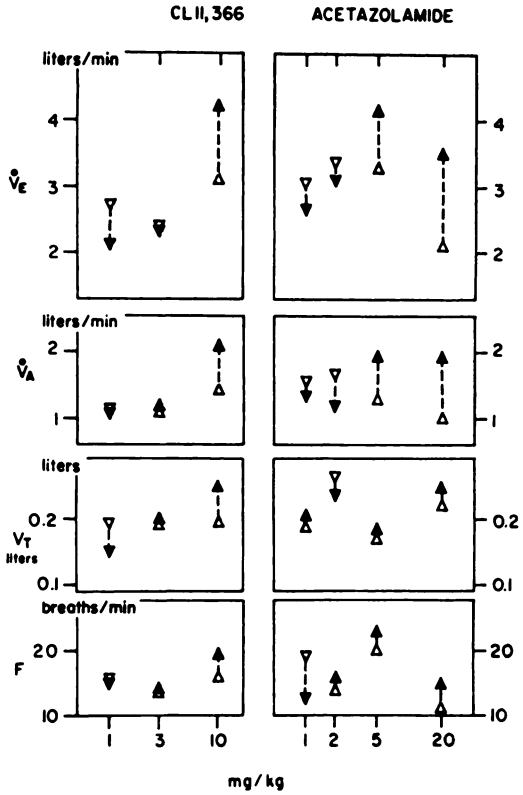


FIG. 6. Ventilatory changes in conscious beagles after CL 11,366 and acetazolamide i.v.

Open triangles represent mean values before drug and solid triangles after drug for the total minute volume of expired gas ($\dot{V}_E$), alveolar ventilation ($\dot{V}_A$), tidal volume of expired gas (V_T) and frequency of breathing (F). Values at 15 and 45 minutes before and after drug were averaged separately for each experiment and the means of these averages shown. For CL 11,366 the number of animals was 2, 5 and 3 at 1, 3 and 10 mg/kg. For acetazolamide the number of animals was 2 except at 2 mg/kg where n = 3.

elicited by 3 mg/kg of CL 11,366, but 10 mg/kg of CL 11,366 gave both a respiratory effect and a CSF pressure response (table 1).

Intraocular pressure, an index of acute changes in formation of aqueous humor, was not altered by 10 mg/kg of CL 11,366 i.v. in two dogs (the pressure changes after drug were within 5% of control values). This result agrees with that of Wistrand *et al.* (1961b) who showed that this dose of CL 11,366 does not lower intraocular pressure in the rabbit. It appeared from those studies that this drug gained access grossly to ciliary structures but was not in equilibrium with enzyme.

A CSF perfusion technique with inulin C^{14} has been used to demonstrate a mean maximal reduction in CSF flow of 40 to 50% following acetazolamide in doses as low as 10 mg/kg (Oppelt *et al.*, 1962). Additional work with CL 11,366 showed that 5 mg/kg gave no change in CSF flow; 10 mg/kg gave 15% reduction in one case and 50% reduction in another; 20 mg/kg gave a full response in 3 dogs.

Postfeeding gastric acid secretion, inhibited by acetazolamide 5 mg/kg i.v. (Byers *et al.*, 1962), was essentially unaltered by CL 11,366 at 3 mg/kg i.v. in 7 experiments in 3 dogs (data not shown).

Respiratory effect and degree of erythrocytic enzyme inhibition. Fractional inhibition of erythrocytic carbonic anhydrase exceeded 0.995 with either drug at maximal fall in $P_{A_{CO_2}}$ and increase in pulmonary ventilation (tables 1 and 3). The estimate of i by equation 1 is based on the assumption that I_0 and E_0 are in complete equilibrium in the red cells. Since part of the inhibitor is bound to nonenzymic receptor as well as E_0 (Maren *et al.*, 1961), calculation of i by equation 1 overestimates the true i. Equation 2 does not involve the use of I_0 in the red cells but rather the RBC_d only and therefore tends to give a lower estimate of i than equation 1 (table 3).

DISCUSSION. *Inhibitor distribution and separation of renal from other physiological responses.* Renal carbonic anhydrase inhibition with maximal rates of urinary HCO_3^- excretion can be obtained in the absence of acute changes in respiration, CSF and intraocular pressure, CSF flow or postfeeding gastric acid secretion by properly chosen doses of CL 11,366 but not by acetazolamide at any dose. The fact that CL 11,366 is a powerful inhibitor of carbonic anhydrase helps to make this possible. CL 11,366 is twelve times stronger than acetazolamide at 37°C (fig. 1).[3] The selective character of the renal action of CL 11,366 contrasts with acetazolamide and is reflected in its distribution; I_0 is higher in kidney and lower in red cells and other tissues with respect to E_0. CL 11,366 is concentrated in the kidney as the result of active secretion by mechanisms for handling acids in renal tubules. During

[3] It is of interest that Hunter and Lowry (1956) obtained a similar K_I for acetazolamide at 37°C and discussed the relationship between degree of inhibition and possible physiological effects.

this process there is apparently full access of inhibitor to carbonic anhydrase (Maren, 1963b).

Relative exclusion of CL 11,366 from red cells is the net result of several factors: 1) This inhibitor is more highly bound to plasma protein than acetazolamide (fig. 1) so that a smaller fraction of drug is available for diffusion from plasma into red cells. 2) CL 11,366 is totally ionized and fat-insoluble at body pH (fig. 1). 3) The diffusible portion of CL 11,366 in red cells is not only small but also decays more rapidly than the diffusible portion of acetazolamide. This decay follows the more rapid fall in plasma inhibitor concentration of CL 11,366 which is secondary to faster renal clearance. 4) Briefly opposing these factors is an initial plasma concentration of CL 11,366 higher than that with equimolar doses of acetazolamide. This is secondary to a lower volume of initial distribution in body fluids but is quickly dissipated by faster renal excretion of this inhibitor.

Separation of renal from erythrocytic responses is further aided by an E_0 in kidney which is only one third that of red cells (Maren, 1962).

Respiratory effects of carbonic anhydrase inhibitors. Two useful indicators of the respiratory effects of the inhibitors are an increase in pulmonary ventilation and a decrease in $P_{A_{CO_2}}$ (Tomashefski *et al.*, 1954). These changes arise from several physiological events which have been summarized in part by Berliner and Orloff (1956). It is apparent from the present work that acute changes in these two indicators are the result of erythrocytic carbonic anhydrase inhibition. The increase in ventilation is probably related to a rise in CO_2 tension in tissues including respiratory centers. The decrease in $P_{A_{CO_2}}$ can be attributed to 1) this hyperventilation and 2) a further reduction in pulmonary capillary CO_2 tension due to interference within red cells of CO_2–carbonic acid exchange.

Mithoefer (1959) demonstrated the individual contribution of these two factors in the decreased $P_{A_{CO_2}}$ by a study of changes in $P_{A_{CO_2}}$ at varying conditions of pulmonary ventilation. He found that the fall in $P_{A_{CO_2}}$ during increased ventilation secondary to acetazolamide was greater than the fall during equivalent artificially induced hyperventilation without drug. Furthermore, he isolated the specific interference with CO_2–carbonic acid exchange in red cells in the pulmonary capillaries by measuring the fall in $P_{A_{CO_2}}$ after drug while ventilation was kept constant at control levels (compare Pocidalo *et al.*, 1958, and Cain and Otis, 1960).

Following the acute respiratory effects of the inhibitors, the renal action becomes important in ventilatory changes. Metabolic acidosis develops as a result of urinary HCO_3^- loss and is incompletely compensated (*e.g.*, table 3). This tends to produce increases in ventilation which persist beyond the initial changes accompanying erythrocytic inhibition (table 3).

During acute erythrocytic inhibition, certain other respiratory measurements are less useful than ventilation and $P_{A_{CO_2}}$. For example, arterial CO_2 tension as ordinarily measured does not clearly indicate the physiological events in either the lungs or peripheral tissues. In fact the arterial values usually obtained overestimate true pulmonary end-capillary CO_2 tension during erythrocytic inhibition (Berliner and Orloff, 1956) and may, of course, grossly underestimate CO_2 tension in the tissues. Therefore the arterial CO_2 tension, which is normally a reliable indicator of alveolar and end-capillary CO_2 tensions in the lung and of alveolar ventilation, has limited usefulness as a guide to ventilatory status during erythrocytic carbonic anhydrase inhibition.

In the conscious animal with ventilatory responses to physiological stimuli intact, erythrocytic inhibition leads to changes in arterial (or venous) CO_2 tensions too small to indicate alone the significant respiratory events which occur. Similarly, the change in CO_2 output and R is transient (compare Carter and Clark, 1958). However, when normal ventilatory responses are impaired sufficiently, blood CO_2 tensions rise while CO_2 output falls. This accumulation of CO_2 in the body occurs during anesthesia in dogs (Tomashefski *et al.*, 1954; Cain and Otis, 1960; Wistrand *et al.*, 1961a) and during artificially controlled ventilation in dog (Pocidalo *et al.*, 1958; Mithoefer, 1959; Cain and Otis, 1960), in normal man and in patients with pulmonary disease (Pocidalo *et al.*, 1960).

Acute erythrocytic inhibition carries the risk of further CO_2 retention in patients with severe lung disease with respiratory failure. Drowsiness and increases in blood CO_2 tensions occurred in patients receiving acetazolamide reported by Nadell (1953) and other workers, reviewed by Berliner and Orloff (1956). Subsequent reports

(see Platts and Hanley, 1956) have emphasized the hazards of acetazolamide therapy in patients with respiratory acidosis.

Elimination of respiratory effects by use of CL 11,366: consequences for experimental studies. The selective properties of CL 11,366 may be utilized to study the relative importance of renal and of erythrocytic inhibitory effects on net physiological responses in various systems of the body. Data are available from the present work for consideration of respiration, cerebrospinal fluid pressure changes, kidney, eye and stomach.

Respiration: Maximal renal HCO_3^- output was obtained at doses of CL 11,366 below 10 mg/kg without eliciting the acute respiratory changes observed at a higher dose. This finding constitutes direct evidence against the early viewpoint of Beckman *et al.* (1940) and others (see summary by Roughton *et al.*, 1941) that changes in pulmonary ventilation and $P_{A_{CO_2}}$ following carbonic anhydrase inhibitors were due only to renal metabolic acidosis. It is apparent that the metabolic acidosis becomes established well after the acute erythrocytic inhibitory effects have appeared (table 3). In addition, any renal effect on ventilation or $P_{A_{CO_2}}$ is much less than the erythrocytic effect.

CSF pressure: In anesthetized dogs there was no CSF pressure rise at doses of CL 11,366 which gave no acute respiratory effect (*e.g.*, 3 mg/kg i.v.). This result supports the conclusion of Mithoefer *et al.* (1957), Knopp *et al.* (1957) and Wistrand *et al.* (1961a) that immediate rise in CSF pressure after i.v. doses of acetazolamide is secondary to inhibition of carbonic anhydrase in red cells.

Kidney: The same renal HCO_3^- response occurred at doses of CL 11,366 with and without erythrocytic respiratory effects. This finding supports the assumption by Höber (1942) and the conclusion of subsequent workers that inhibition of carbonic anhydrase in renal tubules is the predominant if not the sole factor in the magnitude of the observed response.

Eye: Erythrocytic enzyme inhibition has previously received consideration as a potential factor in the net effect of acetazolamide on the lowering of intraocular pressure (Wistrand *et al.*, 1961a). This consideration necessarily arises because of the demonstration that respiratory acidosis induced by CO_2 inhalation lowers intraocular pressure (Wistrand and Maren, 1960), and in certain experimental conditions there may be CO_2 retention with acetazolamide. In such a condition, namely anesthesia, the present work indicated that an effective erythrocytic inhibitory dose of CL 11,366 (10 mg/kg i.v.) was associated with no change of intraocular pressure. Therefore, it is unlikely that inhibition of carbonic anhydrase in red cells is a significant factor in the lowering of intraocular pressure by acetazolamide or by the high doses of CL 11,366 (greater than 10 mg/kg) which are required to gain access to the enzymic site in the ciliary structures of the eye (compare the results and conclusions in the rabbit by Wistrand *et al.*, 1961b, for detailed consideration of the relation between chemical structure, disposition and intraocular effects).

CSF flow: The data show that CSF flow is reduced by CL 11,366 only at doses some 50 times greater than that necessary to produce the renal effect. The reasons may be similar to those just advanced for low activity of this drug on intraocular pressure, based on the likelihood that the intimate structure of the choroid plexus has some similarity to ciliary body.

With respect to a dissociation between carbonic anhydrase inhibition in the red cell and in choroid plexus, the present data do not contribute since approximately the same dose is required for the respiratory effect as for a reduction in CSF flow. However, these two physiological effects have been separated. Oppelt *et al.* (1963) have shown that CO_2 inhalation and the ensuing respiratory acidosis do not affect CSF flow.

Stomach: Postfeeding gastric acid secretion was not inhibited by CL 11,366 (3 mg/kg i.v.), probably due to lack of access of inhibitor to enzyme in gastric mucosa at this dose. Byers *et al.* (1962) observed a period of augmented acid secretion after an initial phase of inhibition of secretion with acetazolamide. Metabolic acidosis was regarded to be the chief factor in this period of hypersecretion of acid. However, a period of augmented secretion of acid was absent after CL 11,366 even though metabolic acidosis was present. Two explanations are tentatively offered for this result: 1) at the dose of CL 11,366 used in these experiments, the acute erythrocytic respiratory effect was absent and may therefore be involved in the elevated acid production observed with acetazolamide, and 2) the metabolic acidosis in these particular experiments

with CL 11,366 may have been somewhat less than in the work of Byers *et al.* (1962).

Relation between enzyme inhibition and physiological effect. The value of i at minimal doses of inhibitor for maximal response is about the same (greater than 0.995) for kidney and red cell (table 1). The data for red cell and respiratory changes support previous estimates of the degree of inhibition generally required for physiological effect (Maren, 1963b). Calculations of i by equation 2 appear to substantiate the provisional conclusion, based on CSF pressure changes after methazolamide and acetazolamide (Wistrand *et al.*, 1961a), that RBC_d is in equilibrium with carbonic anhydrase and therefore determines the degree of inhibition.

SUMMARY

Carbonic anhydrase inhibitors including acetazolamide usually elicit physiological responses at various sites in the body without discrimination by dose. These responses, particularly those related to erythrocytic enzyme inhibition, tend to complicate study of any single system. A compound related to acetazolamide, 2-benzenesulfonamido-1,3,4-thiadiazole-5-sulfonamide (CL 11,366), was found to have properties which make possible a separation of renal from erythrocytic and other effects. Acetazolamide and CL 11,366 were given intravenously to dogs over a wide range of dose. Studies were made of inhibitor distribution and physiological responses in kidney, red blood cells and certain other tissues.

Acetazolamide at the lowest dose which gives maximal renal HCO_3^- output (10 mg/kg) gave acute respiratory changes including hyperventilation and a fall in alveolar CO_2 tension. This is a dose known to be associated with other responses such as an acute rise in cerebrospinal fluid pressure, decreases in cerebrospinal fluid flow and intraocular pressure, and inhibition of post-feeding gastric acid secretion. By contrast, CL 11,366 was not accompanied by respiratory or the other responses over a range of dose which gave a full renal response (0.3 to 3 mg/kg). Effective erythrocytic enzyme inhibition with respiratory changes occurred at 10 mg/kg.

Elimination of acute respiratory effects during maximal renal carbonic anhydrase inhibition by CL 11,366 is related to 1) partial exclusion of inhibitor from red cells by high plasma binding, ionization and rapid plasma decay, and 2) concentration of inhibitor in kidney by active secretion. Acute respiratory changes were demonstrated to be related to inhibition of erythrocytic carbonic anhydrase (greater than 0.995) independent of the renal HCO_3^- loss.

The results indicate that selective renal carbonic anhydrase inhibition without acute effects on respiration can be obtained by the use of appropriate doses of CL 11,366.

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