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THE CARBONIC ANHYDRASE INHIBITOR IN SERUM

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An active inhibitor of carbonic anhydrase found in the sera of pig and other mammals, was briefly described in a recent note [Booth, 1936]. Carbonic anhydrase is normally found only in corpuscles, and its absence from the plasma has distinct advantages in the regulation of the blood *p*H *in vivo* [Roughton, 1935]. The function of the inhibitor was thought to be a protective one in that it provides a temporary mechanism for ensuring that any carbonic anhydrase which may be set free into the circulating plasma as a result of corpuscle destruction does not annul these advantages. Much of the force of this suggestion was lost on finding that the inhibitor is not present in the blood of man. However, even though the inhibitory activity of serum may be accidental it was considered of interest to study (*a*) the kinetic relations of the inhibitor to the enzyme, (*b*) its own chemical nature, and (*c*) its distribution.

Methods

The dehydration rates of carbonic acid were determined with the apparatus of Meldrum & Roughton [1933 *a*] in which bicarbonate solution is suddenly mixed with phosphate buffer and the CO_2 evolution is observed manometrically. The hydration rates (CO_2 uptake by buffer) were determined in a similar apparatus, as described by Meldrum & Roughton [1933 *b*], but with certain modifications. The technique is essentially the same as that used for measuring CO_2 output rates except that CO_2 was added to the manometer vessel (or "boat") and the rate of change of pressure due to its uptake by buffer solution observed manometrically. The boat was first evacuated to 0·1 atm.: then 2 ml. CO_2 at 1 atm. were introduced into the 60 ml. gas space, making the final concentration of CO_2 25 p.c. at 0·133 atm.

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To estimate the inhibitory power of a solution four runs were necessary: (1) the control; (2) the same with the addition of enzyme to the phosphate buffer; (3) with enzyme and test solution; (4) with test solution but without enzyme. The fourth run was made to determine whether the test solution itself contained enzyme. In the case of CO_2 output the accelerations were calculated from the reciprocals of the times taken for the second quarter of the process [Meldrum & Roughton, 1933*a*]. In the case of CO_2 uptake the curves are diphasic, the initial rapid phase being due to physical solution of CO_2. The rates were expressed as the slopes of the second phases. If R_0, R_1, R_2 represent the control, enzyme and inhibited enzymic rates respectively, the enzymic acceleration A_1 in absence of inhibitor is $(R_1-R_0)/R_0$ and in presence of the inhibitor is $A_2=(R_2-R_0)/R_0$. The percentage inhibition is then $(A_1-A_2)\times 100/A_1$.

In CO_2 evolution experiments an inhibitor effect is to some extent simulated by alkali. Hence all test solutions were adjusted to *p*H 7·4. In doubtful cases the genuineness of the inhibitory effect could be checked by making use of the following three facts: (1) alkali decreases the total CO_2 evolved; (2) alkali slows the control rates; (3) alkali accelerates CO_2 uptake (enzymic) while the serum inhibitor slows it. The converse holds throughout in the CO_2 uptake experiments.

Material

Enzyme solutions were prepared from washed pig or horse corpuscles by Meldrum & Roughton's [1933*a*] chloroform method. For certain experiments the solution obtained by laking corpuscles with distilled water was used as source of enzyme without further purification. Serum was used as fresh as possible. When dilution was necessary saline was added.

Kinetics

Inhibitor concentration curves. To constant quantities of pig enzyme solution graded amounts of pig serum were added and made up to a standard volume. Aliquot portions of each mixture were added to the phosphate in the boat and tested (see Fig. 1). In absence of serum the quantity of enzyme used in these experiments accelerated dehydration 3·2-fold. A similar series of curves was also obtained for horse enzyme (chloroform preparation) and pig serum. From the former curves the percentage inhibition was calculated, and Fig. 2 shows how the degree of inhibition depends on the concentration of serum. The enzyme was inhibited almost 90 p.c. by 0·05 ml. serum (with 4·95 ml. total fluid in

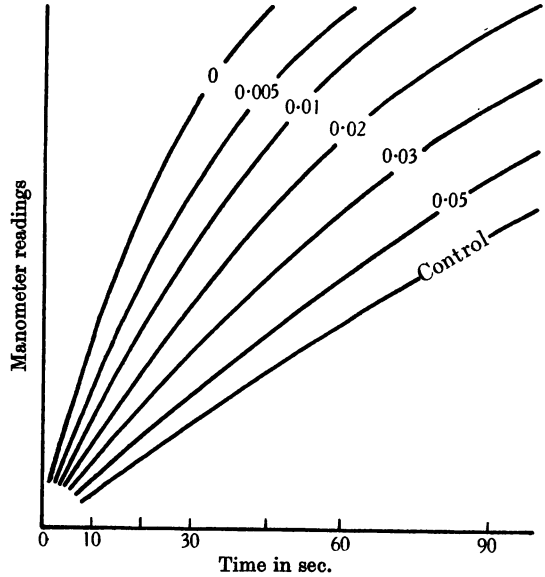

Fig. 1. Inhibition of carbonic anhydrase by pig serum. CO_2 output. 19°. 4 ml. 0·2 *M* phosphate *p*H 7·4 + 0·25 ml. 0·2 *M* bicarbonate + 0·1 ml. enzyme solution; ml. serum as shown by figures on the curves; saline to make total volume up to 4·95 ml. The control without enzyme was also the same when 0·05 ml. serum was added. Maximum acceleration without serum 3·2.

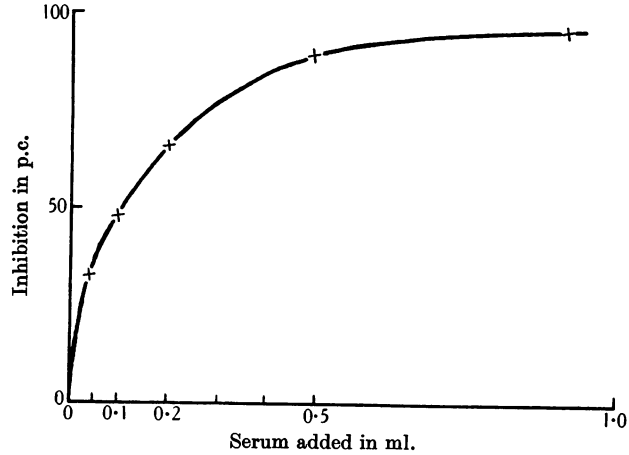

Fig. 2. Percentage inhibition curve. Enzyme constant; serum varied. 19°. Plotted from Fig. 1.

the boat, making the final dilution of serum 1 in 100); and 50 p.c. by 1 in 500. The control curve in Fig. 1, representing the uncatalysed rate, is the same whether serum is added or not. In fact no case of slowing of the control velocity has yet been found. Serum therefore affects the enzyme rather than the chemical reaction itself.

Graded amounts of enzyme were tested with and without serum, the concentration of serum being maintained constant at a final dilution of 1 in 500 (see Fig. 3). The stronger the enzyme solution the lower is the percentage inhibition due to a given amount of serum. Since the range of activity over which the apparatus gives accurate results is narrow the curve is necessarily limited, the value for the percentage inhibition for the highest enzyme activity being least accurate.

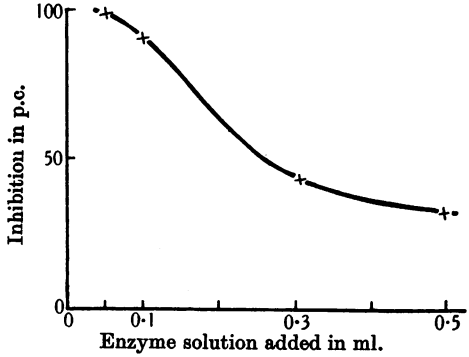

Fig. 3. Percentage inhibition curve. Serum constant; enzyme varied. CO_2 output. 19°. Quantities as for Fig. 1, with 0·1 ml. serum. 0·1 ml. enzyme without serum accelerated 1·6.

Pig serum sometimes contains demonstrable amounts of enzyme. Since the activity of the enzyme must be partly suppressed by the inhibitor normally present, such serum must actually contain more enzyme than is indicated. The activity of any further enzyme added is also found to be partly suppressed, as would be expected from a consideration of Fig. 3. The serum used in the above experiments, although it showed no catalytic activity, may have contained small amounts of enzyme. The true inhibition curve (Fig. 2) would therefore more nearly resemble a rectangular hyperbola. Similar considerations apply to Fig. 3. There would be more enzyme present when serum was added than in its absence: hence the percentage inhibition must have been greater than was observed.

A series of runs was made with graded amounts of a chloroform enzyme preparation (human) when the concentration curve *A* in Fig. 4

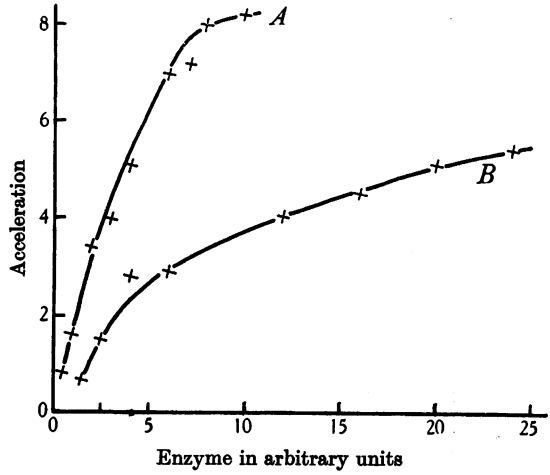

Fig. 4. Comparison of enzyme concentration curves with and without serum. CO_2 output. 14°. Quantities as for Fig. 1. *A*, enzyme alone; *B*, with serum: enzyme and serum both varied but ratio constant. Human chloroform enzyme preparation. Pig serum.

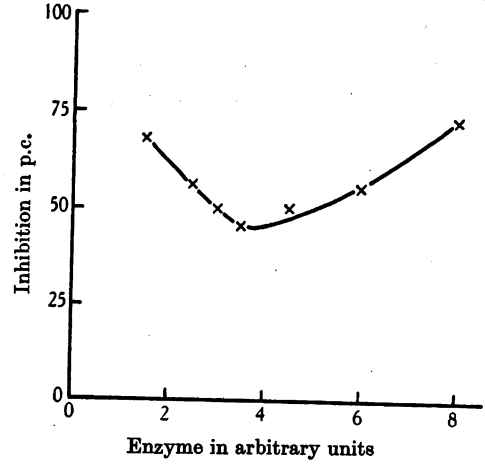

Fig. 5. Percentage inhibition curve. 14°. Enzyme and serum varied together. Plotted from Fig. 4.

was obtained. To this same enzyme solution pig serum was added in such amount as to halve a normal fourfold inhibition, 1 vol. serum being required for 5 vol. enzyme solution. A series of runs was made using

from 0·04 to 0·6 ml. of this enzyme-serum mixture. The concentration curve obtained is shown for comparison in Fig. 4. One essential difference is that the acceleration in absence of serum reaches a maximum of 8·2 and in presence of serum of only 6. The diffusion rates from liquid to gas phase in the apparatus probably set these limits to the observed accelerations. Serum proteins may interfere with this diffusion. At any rate, certain other proteins lower this maximum acceleration, although they show no inhibitor effect at moderate enzyme concentrations. From these two enzyme concentration curves (with and without inhibitor) the percentage inhibition for constant enzyme/serum ratio has been calculated and plotted in Fig. 5. The minimum inhibition was obtained with an enzyme concentration which accelerated (in absence of inhibitor) 4·5-fold. Again it becomes clear that for a given concentration of inhibitor the percentage inhibition is greater the lower the enzyme strength. When the acceleration is much less than 1, however, the experimental method becomes unreliable.

Therefore when testing any material for the presence of inhibitor the enzyme concentration has usually been adjusted to produce an acceleration of between 1 and $2\frac{1}{2}$.

Effect of prolonged action of serum on the enzyme. The effect of prolonged interaction between the enzyme and serum was investigated. The enzyme solution accelerated 3·2-fold, and the serum added had a final dilution of 1 in 500, producing 50 p.c. inhibition. Curves *E* and *A* (Fig. 6) represent the rates with and without enzyme respectively. An enzyme-serum mixture was tested at once and again after $4\frac{1}{2}$ and 7 hr. respectively. The several curves (*B*) coincided exactly, showing that the phenomenon is a genuine inhibition and not due to destruction of enzyme. The same amounts of enzyme and of serum were also added directly to the buffer in the boat, without previous mixing, and tested after the shortest practicable equilibration period. The rate, as shown by curve *C*, is minutely faster than rate *B*. For *D* the enzyme was added to the buffer and the serum to the bicarbonate: thus inhibitor and substrate came into contact with the enzyme simultaneously. The rate here was a little greater initially but after 20 sec. was the same as for *B*.

Inhibition of hydration. Fig. 7 shows the effect of serum on CO_2 uptake by phosphate buffer *p*H 7·4. The amount of enzyme solution used accelerated CO_2 uptake 2·6-fold. When 0·02 ml. serum was added (total fluid 4·20 ml.) the acceleration fell to 0·26-fold—that is, an inhibition of 90 p.c. was obtained by 1 in 210 serum. This is in reasonable agreement with the values for the inhibition of CO_2 output.

Effect of phosphate concentration. The CO_2 uptake method at low pressure allows variation of the concentration of the phosphate buffer. In experiments carried out with Dr F. J. W. Roughton the control rate was found to increase with the phosphate concentration. Later I tested the effect of serum on the enzyme at two phosphate concentrations at 17°

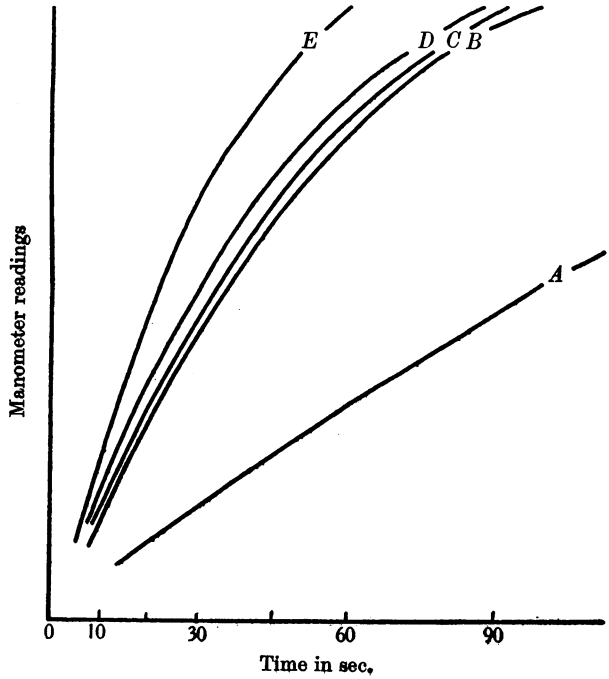

Fig. 6. Effect of prolonged contact between enzyme and serum. CO_2 output. 18°. Quantities as for Fig. 1. *A*, control without enzyme; *E*, enzyme without serum; *B*, *C*, *D*, same amount of enzyme as for *E* but with 0·01 ml. serum added in various ways: see text for details.

and *p*H 7·3. Pig serum (1 in 110) depressed the enzyme-accelerated rates with 0·2 *M* and 0·02 *M* phosphate by 85 and 75 p.c. respectively. Serum had no effect on the control rate at either phosphate concentration. Most of the experiments in this paper (whether CO_2 output or uptake) were done with about *M*/6 phosphate: the concentration was never varied within one experiment.

Effect of temperature. Temperature had an unexpected effect on the efficacy of serum as inhibitor. The experimental results are summarized

in Table I. It is clear that a smaller amount of serum has a greater inhibiting effect on a larger amount of enzyme at 36° than at 0°.

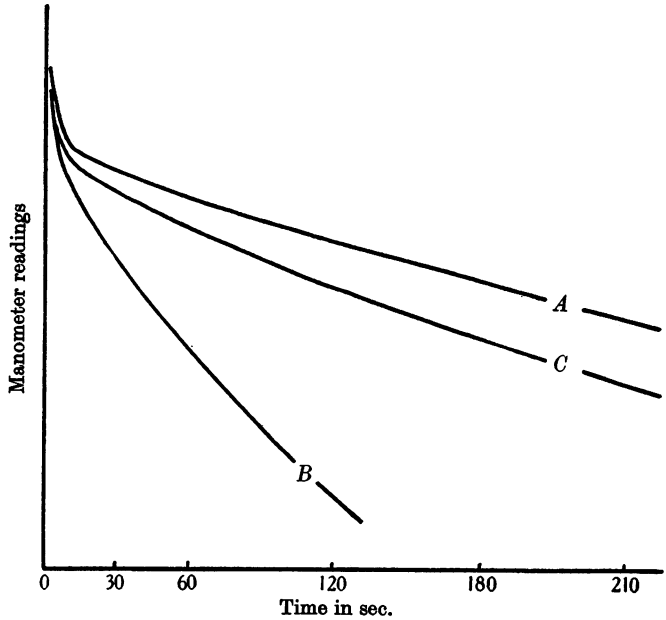

Fig. 7. Inhibition of carbonic anhydrase by serum. CO_2 uptake. 0°. 4 ml. 0·2 *M* phosphate *p*H 7·4 + 0·2 ml. enzyme, serum and/or saline. 25 p.c. CO_2 in 0·133 atm. total pressure. *A*, control; *B*, with enzyme; *C*, with enzyme + 0·02 ml. serum.

TABLE I. Effect of temperature on serum inhibitor

CO_2 uptake. 25 p.c. CO_2 in total pressure of 0·133 atm. 5 ml. 0·023 *M* phosphate *p*H 7·3. Pig serum and 1 in 200 pig chloroform enzyme solution as shown. Water to 5·5 ml.

Temperature ° C.	Enzyme solution ml.	Serum ml.	Inhibition p.c.
0	0·06	0·05	65
17	0·12	0·05	80
36	0·4	0·02	80

Effect of p*H*. Fig. 8 shows the *p*H activity curves with and without enzyme, and with enzyme and serum both present. The percentage inhibition by 1 in 1340 serum (calculated from *C*, *B* and *A*) is plotted in Fig. 9. Although the activity of the enzyme increases with rise in *p*H, the increase in the effectiveness of the serum inhibitor is even greater, as shown by the rise in the p.c. inhibition curve. In another experiment using three times as much serum the inhibition was approximately constant at 90 p.c. irrespective of *p*H.

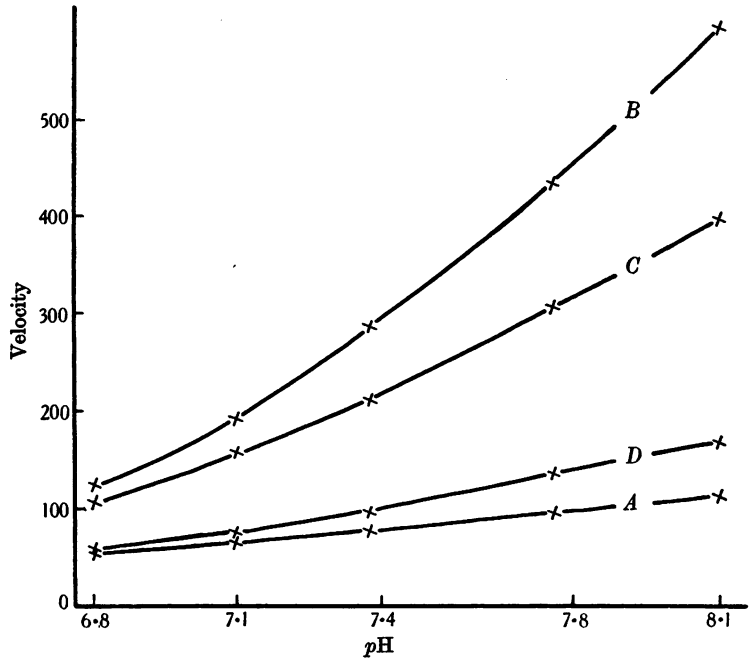

Fig. 8. Effect of *p*H. Enzyme and serum both constant; *p*H varied. CO_2 uptake. 0°. 25 p.c. CO_2 in 0·133 atm. total pressure. 4 ml. 0·2 *M* phosphate buffer + 0·2 ml. water or other solution. Pig chloroform enzyme preparation. Pig serum. *A*, control without enzyme; *B*, with enzyme; *C*, with enzyme + serum 1 in 1340; *D*, with enzyme + serum 1 in 420.

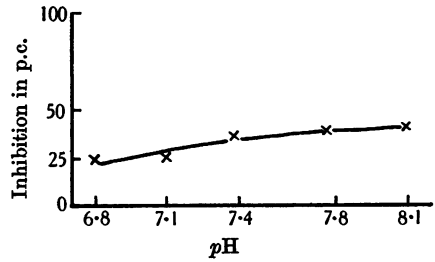

Fig. 9. Percentage inhibition curve. Enzyme and serum (1 in 1340) both constant; *p*H varied. Plotted from Fig. 8.

The nature of the inhibitor

The chemical nature of the inhibitor was studied by fractionating serum and testing each fraction on a horse enzyme solution.

Dialysis. Fresh pig serum was dialysed for 48 hr. in cellophane tubes, first against running tap water, then against two changes of distilled

water. The temperature averaged 10°. The volume increased by about 50 p.c. and a precipitate of euglobulin formed. An equal volume of distilled water was added, the precipitate centrifuged off and dissolved in a small amount of saline. This solution inhibited, but to a much smaller extent than the original serum. Most of the inhibitor remained in the supernatant solution: little or none had either dialysed or precipitated out. This dialysed, diluted and centrifuged serum was used for several of the experiments which follow. A dialysis experiment was also arranged in another way. A narrow (1 cm.) cellophane tube containing 10 ml. distilled water was suspended in 500 ml. fresh pig serum. The serum was stirred and the tube shaken occasionally at room temperature over a period of 48 hr. If the inhibitor is at all dialysable this large excess of serum over water should provide excellent conditions for its removal by dialysis. When the solution from the dialysing sac was tested, however, it contained no inhibitor.

Precipitation. A sample of diluted, dialysed, euglobulin-free serum was half saturated with ammonium sulphate. The precipitate was centrifuged off and the supernatant solution found to be only slightly active. The precipitate was washed with half-saturated ammonium sulphate solution, dissolved in water and reprecipitated. An aqueous solution of this last precipitate was very active. Euglobulin, obtained from fresh serum by precipitating by one-third saturation with ammonium sulphate, was found to inhibit only to a small extent. On the other hand the washed and neutralized precipitates obtained by adding one-third of its volume of 5 p.c. hydrochloric acid to serum, or by saturating with magnesium sulphate, were very active. Thus most, but not quite all, of the inhibitor followed the pseudoglobulin fraction. Neither magnesium nor ammonium sulphates or chlorides themselves inhibited carbonic anhydrase.

Denaturation. Fresh serum was boiled at *p*H 5·4 for 3 min. The coagulum was filtered off and suspended in saline. Both the filtrate and the suspension were adjusted to *p*H 7·4 and tested. Neither showed any inhibition. To a portion of the dialysed, diluted and centrifuged serum from above an equal volume of water was added (corresponding to serum diluted 1 in 5) and a few drops of alkali. This solution was boiled for 4 min. to convert the protein to metaprotein. No precipitate formed but the solution became opalescent, particularly on cooling and adjusting to *p*H 7·4 for testing. It was moderately active.

Other protein denaturing processes were also applied to serum. 1 vol. cooled dialysed serum was added to 2 vol. cooled ethyl alcohol and allowed to stand at 2° for 1 hr. This treatment completely and irreversibly

precipitates all the protein, which can be filtered off leaving an a-biuret filtrate. The precipitate, which was dried, suspended in saline and tested at once, was inactive. The filtrate was also inactive after the alcohol had been distilled off *in vacuo*. The experiment was repeated with acetone. The a-biuret filtrate was inactive. The precipitate inhibited if dissolved and tested at once but not if allowed to dry. Acetone itself, used as a control, did not affect the enzyme. Alcohol denatures serum proteins and converts them to an insoluble form resembling a heat coagulum. Acetone also denatures and completely precipitates the proteins but the precipitate only becomes insoluble on drying.

Association with protein. These experiments are summarized in Table II. The inhibitions have not been expressed as percentages and are

TABLE II. Solubility of the inhibitor. Serum fractions tested on enzyme from horse blood

Treatment applied to serum		Ppt. dissolved or suspended in salt solution *p*H 7·4	Filtrate or centrifugate
1. Dialysis and dilution	s	+	+ + + +
2. Dialysate	.	.	0
3. Acidified with HCl	s	+ + +	+ +
4. 1/3 saturated $(NH_4)_2SO_4$	s	+	+ + + +
5. 1/2 saturated $(NH_4)_2SO_4$	f	+ + + +	+
6. 1/2 saturated $(NH_4)_2SO_4$	d	+ + + +	+
7. Saturated $MgSO_4$	d	+ + + +	+
8. Boiled at *p*H 5·4	s	0	0
9. Boiled at *p*H >8	d	No ppt.	+
10. Added alcohol at 2°	d	0	0
11. Added acetone at 2°	d	.	0
Ppt. not dried	.	+ +	.
Ppt. dried	.	0	.
12. Controls: $(NH_4)_2SO_4$	.	.	0
$MgSO_4$	.	.	0
NaCl	.	.	0
Acetone	.	.	0

s = fresh serum.
d = dialysed serum.
f = solution from Exp. 4.

+ = inhibitor present.
0 = inhibitor absent.

not all strictly comparable. Nevertheless certain conclusions can be drawn as to the nature of the inhibitor.

(1) It is not dialysable.

(2) Exps. 7–10 indicate that its activity is bound up with the solubility of the serum proteins. When the proteins are rendered less soluble as by converting to metaprotein, or insoluble as by coagulating, the inhibitor is respectively weakened or destroyed.

(3) It is always precipitated with the protein. Much the greatest proportion follows the pseudoglobulin, while the euglobulin and albumin fractions (or at least the fraction precipitated by one-third, and the

fraction not precipitated by one-half, saturation with ammonium sulphate) show comparatively little activity. There is some evidence [Chick, 1914; Rimington, 1933] that euglobulin is (wholly or partially) denatured pseudoglobulin: in that case the decrease in activity accompanying denaturation is in agreement with the lower activity of euglobulin. It is possible that some protein closely resembling pseudoglobulin may not be completely precipitated by half saturation with ammonium sulphate and that this accounts for the activity of the "albumin" fraction.

Although the evidence for the protein nature of the inhibitor is not complete there can be little doubt that it is closely associated, if not identical, with serum globulin.

Is the inhibitor an artifact?

For most of the work on the inhibitor a "crude chloroform preparation" was kept in the refrigerator and used over a period of several days, whereas the serum was generally used fresh. Enzyme solutions prepared by laking corpuscles from various sources with distilled water were also inhibited by sera from several animals. Pig serum frequently contains visible amounts of hæmoglobin, and presumably therefore carbonic anhydrase, yet no enzyme activity can be demonstrated. This can only mean that the serum inhibitor completely suppresses enzyme activity at this crude stage of purity as well as at the more advanced stages. In more than one experiment an enzyme preparation from pig blood was found to be inhibited by serum from the *same specimen* of blood: hence there can be no question of a "foreign protein" effect.

Since serum differs from plasma the results were checked with plasmas obtained by mixing freshly drawn blood with (*a*) heparin, and (*b*) potassium oxalate, and centrifuging. Both plasmas inhibited to the same extent as serum. Oxalate itself had no effect on the enzyme. It is clear that the inhibitor is not formed during the production of serum. Indeed there is no reason to disbelieve that the inhibitor exists in the blood *in vivo*.

Distribution

Mammalian sera. The sera or treated plasmas from various mammals and from birds have been tested (see Table III). While the inhibitor is found in the sera of several domesticated mammals, it is noteworthy that human blood contains none. Nor is any found in the sera from monkey, duck, or pigeon. The tests on the distribution of the inhibitor were performed at room temperature. Because the activity of pig serum is greater

TABLE III. Distribution of the inhibitor

Material	Carbonic anhydrase	Enzyme preparation used	Inhibitor
Pig serum	0	h	+ + + + + +
Pig serum	.	m	+ + + + + +
Pig serum	.	p	+ + + + + +
Pig heparinized plasma	0	p	+ + + + + +
Pig oxalated plasma	.	p	+ + + + + +
Pig serum and oxalate	.	p	+ + + + + +
Human serum	0	p	0
Human serum	.	m	0
Human heparinized plasma	.	m	0
Human oxalated plasma	0	p	0
Human oxalated plasma	.	m	0
Human jaundiced serum (1)	.	p	0
Monkey serum (2)	0	p	0
Ox serum	0	p	+ +
Horse serum (heated)	.	p	+ +
Cat oxalated plasma	.	p	+ + + + +
Rat serum	.	p	+ + + + + +
Rat serum	.	m	+ + + + +
Sheep serum	.	p	+ + +
Sheep fœtal serum (85 day) (3)	.	m	+ + +
Rabbit maternal oxalated plasma	Trace	r	+
Rabbit fœtal oxalated plasma	Trace	e	+
Guinea-pig serum	.	p	+
Fowl whole plasma (5)	0	p	Trace
Fowl whole plasma (5)	.	f	Trace
Fowl serum (5)	.	f	Trace
Duck serum (4)	0	m	0
Pigeon serum	0	ox	0
Human urine	A	p	0
Cat urine	0	ox	0
Cow's milk	A	p	0
Pig bile	Trace	p	0
Human saliva	.	p	0
Pig stromata	Trace	p	+
Witte's peptone	.	p	+
Globulin from cow's whey	.	p	Trace
Snail blood (*Helix pomatia*) (6)	0	p	0
Maia blood (6)	0	p	0
Hamlet serum (7)	Trace	p	Trace
Octopus blood (7)	.	p	0
Commercial blood albumin	.	h	+
Commercial egg albumin	.	p	0
Fresh egg-white	.	p	Trace
Glaucoma pyriformis, whole (1)	0	.	—
Glaucoma pyriformis, disintegrated (1)	0	p	Trace
Baker's yeast suspension	A	p	0
Potassium oxalate (as control)	.	p	0

Explanation of signs

Material (1) supplied by Dr N. W. Lawrie, (2) by Prof. E. D. Adrian, (3) by Sir Joseph Barcroft, (4) by Dr C. Ludwig, (5) by Mr E. N. Willmer, (6) by Dr E. Baldwin, (7) by Dr F. J. W. Roughton.

+ = inhibitor (or enzyme) present.
0 = inhibitor (or enzyme) absent.
A = enzyme shown absent by Meldrum & Roughton [1933*a*].
h = horse chloroform enzyme preparation.
m = human chloroform enzyme preparation.
ox = ox chloroform enzyme preparation.
p = pig chloroform enzyme preparation.
f = fowl laked corpuscle solution.
r = rabbit laked corpuscle solution.
e = rabbit fœtus laked corpuscle solution.

at 36° than at 17° human serum was also tested at 37°. No inhibition was observed.

Meldrum & Roughton [1933*a*] compared the concentration of enzyme in fœtal and maternal goat blood. They found the former was weak until about 4/5 full term when it rapidly approached the maternal value. It was of interest to know whether the inhibitor makes its appearance simultaneously. One specimen of fœtal sheep serum was obtained from an 85-day fœtus. This is about 3/5 full term. The fœtal serum showed about the same activity as that from an adult. Rabbit serum inhibited only slightly, but here too the fœtal serum was as effective as the maternal.

Miscellaneous biological material. Certain other physiological fluids have been tested. The results appear in Table III. Milk, urine, and bile from animals whose sera are active contain no inhibitor. Bloods from certain lower animals which contain neither corpuscles nor carbonic anhydrase also contain no inhibitor. Various preparations from mammalian tissues were tested. Globulin was precipitated from cow's whey by half saturating with ammonium sulphate. This material contains a powerful xanthine oxidase which inhibits certain enzymes, e.g. amino acid oxidase [Keilin & Hartree, 1936], and glucose dehydrogenase [Harrison, 1933]; it also contains nucleosidases which destroy cozymase [Booth, unpublished]. The inhibiting effect of this globulin on carbonic anhydrase was feeble. Stromata were prepared from pig corpuscles, washed twenty times and suspended in saline. The preparation has some slight enzyme activity and a small though definite inhibitory effect.

Two samples of *Glaucoma pyriformis* were tested. One was a suspension of the living protozoon and the other had been disintegrated by repeated freezing and thawing. Neither contained any carbonic anhydrase but the latter inhibited pig enzyme slightly.

When a trace of enzyme was found in any preparation this was frequently accompanied by measurable amounts of the inhibitor. Although the latter does not completely inhibit the enzyme already present it partially inhibits any extra enzyme which is added. This agrees with the finding in the section on kinetics, that percentage inhibition varies with the strength of enzyme.

In mammals the inhibitor is probably confined to the blood (and lymph) although a definitive conclusion must await further results. Certain other biological materials show barely measurable inhibitory effects: this, however, may be due to physical rather than chemical causes.

Discussion

The fact that the inhibitor is absent from the bloods of certain animals (man and monkey in particular) weakens the force of the original suggestion that its function is a protective one. However, it is of interest to evaluate the possible extent of this protection in the case of the pig. At 17° 0·05 ml. serum inhibits 0·1 ml. of 1 in 200 laked blood 90 p.c. and the inhibitor is nine times as effective at 37°. The serum from 100 ml. pig blood (amounting to 55 ml.) would inhibit (to 90 p.c.) the enzyme from $\frac{55}{0{\cdot}05} \times \frac{0{\cdot}1}{200} \times 9 = 4{\cdot}95$ ml. blood. That is, the inhibitor in circulating blood could only effectively inhibit the enzyme liberated by lysis of at most 5 p.c. of the corpuscles. We may reasonably assume a mean life of 25 days for corpuscles. A less certain but not unreasonable assumption is that not more than one-quarter of those undergoing destruction do so by fragmentation in the blood. About 1 p.c. of the total enzyme would then be liberated into the plasma per day. The inhibitor could suppress 90 p.c. of 5 days' accumulation.

The experiments described in this paper prove that the inhibitor can function *in vitro* but we know nothing of its effect *in vivo*. Even if the inhibitor does perform the suggested function it can at most only be a temporary one and in a limited number of species. The mechanism of the final disposal of the enzyme from the tissues remains unknown.

Summary

1. An active inhibitor of carbonic anhydrase has been found in the sera from pig, sheep, horse, ox, cat, and rat, in oxalated and heparinized plasmas and in fœtal sera.

2. The inhibitor is absent from the bloods of man, monkey, duck and pigeon.

3. The inhibitor is not found in milk, urine, or bile from animals whose sera inhibit, nor in blood (of certain lower animals) which contains no carbonic anhydrase.

4. The inhibitor has many of the properties of pseudo-globulin from which it has not been separated.

5. Serum has no effect on the uncatalysed rates of CO_2 uptake or output but inhibits the enzymic acceleration of both to about the same degree.

6. Percentage inhibition increases with serum concentration, and for a given amount of serum, the inhibition decreases with increasing amount

of enzyme. The inhibition is not increased by prolonged contact between enzyme and serum and hence is not due to destruction of enzyme.

7. The inhibition increases with rise in temperature but is only slightly affected by *p*H.

8. The inhibitor in pig blood could suppress 90 p.c. of the enzyme liberated from about 5 p.c. of the corpuscles.

9. The originally suggested protective function is discussed in the light of the absence of the inhibitor from human blood.

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