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CARBONIC ANHYDRASE IN THE LENS AND IN THE CILIARY BODY AND IRIS OF ALBINO RABBITS

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The presence of carbonic anhydrase in intra-ocular tissues has been reported by earlier investigators whose results are summarized in Table 1. Interest in these findings has been increased by recent reports that the intra-ocular pressure may be reduced both in glaucomatous eyes (Becker, 1954) and in normal eyes of animals (Grant & Trotter, 1954) by the systemic administration of a carbonic anhydrase inhibitor (Diamox, 2-acetylamino-1:3:4-thiadiazole-5-sulphonamide). It was, therefore, decided to investigate further the carbonic anhydrase content of the ciliary body and iris and of the lens, and to discover approximately the relative amounts of enzyme in these tissues.

Table 1. The occurrence of carbonic anhydrase in ocular tissues

Sclera	*Mammals—trace (Bakker, 1941)	—	—
Cornea	*Mammals—small amount (Bakker, 1941)	—	—
Choroid	Fishes—present (Leiner, 1940)	*Mammals—small amount (Bakker, 1941)	—
Ciliary body	Birds—small amount (Kauth & Sommer, 1953)	Rabbits—present (Wistrand, 1951)	Rabbits—present (Green *et al.* 1954)
Iris	—		
Retina	Fishes—present (Leiner, 1940)	*Mammals—present (Bakker, 1941)	Birds—present (Kauth & Sommer, 1953)
Lens	Rats—large amounts (Bakker, 1939)	*Mammals—large amounts (Bakker, 1941)	Birds—present (Kauth & Sommer, 1953)
Aqueous humour	Species not stated—absent (v. Goor, 1934)	—	—
Vitreous body	*Mammals—trace (Bakker, 1941)	—	—
Pecten	Birds—large amounts (Kauth & Sommer, 1953)	—	—

* Rabbits, pigs, cats, sheep, dogs, horses.

Demonstration of the enzyme in avascular tissues, e.g. the lens, presents few difficulties, but in the vascular ciliary body and iris the investigation is complicated by the high carbonic anhydrase content of erythrocytes. To overcome this difficulty the tissue must be perfused with saline to minimize its blood content. Some workers, e.g. Wistrand (1951), have used the benzidine reaction to estimate the blood content of the extracts, while others, e.g. Kauth & Sommer (1953), have assumed that extracts prepared from perfused tissues are practically blood-free. The latter appears a dangerous assumption if the presence of small amounts of enzyme is being investigated. Preliminary experiments showed also that the benzidine reaction, in certain circumstances, could give rise to misleading estimates of the blood content of extracts prepared from the ciliary body and iris.

METHODS

Perfusion

Albino rabbits (weighing 2·0–3·5 kg) were anaesthetized by the intravenous injection of a 25% solution of urethane in doses of 1·75 g/kg. Both jugular veins and both carotid arteries were exposed. 1 ml. of heparin solution (1000 i.u./ml.) was injected intravenously and 5–10 ml. of blood were then withdrawn by heart puncture. Cannulae were inserted into both carotid arteries, and the jugular veins were then severed and the heart excised. Physiological saline (prepared in glass-distilled water) was perfused through the carotid cannulae at a pressure of 1 m of saline. After 1 l. of saline had been perfused, needles were inserted into the anterior chamber of each eye and a pressure of 50 cm of saline applied for several minutes, after which the eyes were enucleated.

Preparation of tissue extracts and blood dilutions

(*a*) *Ciliary body and iris.* Immediately after enucleation the eyes were bisected equatorially. The anterior segment was turned inside out and, by grasping the vitreous with forceps, the vitreous and lens together were removed from the other tissues. Any small fragments of vitreous adhering to the ciliary body were carefully removed. The ciliary body was severed from the choroid with fine scissors and by gentle traction the ciliary body and iris were removed. Excess moisture was removed with filter-paper, the tissues were weighed and then ground up in a glass tissue-grinder with glass-distilled water. The extract was centrifuged and the supernatant liquid kept. The ciliary bodies and irides from both eyes of each rabbit were ground up with 5 ml. of water for use in the manometric determinations and with 10 ml. of water for the indicator method.

(*b*) *Lens.* Any vitreous adherent to the lens was removed and excess moisture was removed with filter-paper. Both lenses from each animal were weighed together and ground up with sand and 10 ml. of glass-distilled water. The resulting fluid was centrifuged and the supernatant liquid kept.

(*c*) *Blood.* From the blood obtained by heart puncture various dilutions between 1:50 and 1:100,000 were prepared with glass-distilled water.

(*d*) *'Heated' extracts.* These were prepared by heating in a water-bath at 70° C for about 60 min.

Manometric estimations of carbonic anhydrase activity

A modification of the method of Krebs & Roughton (1948), using Warburg manometers, was employed. The determinations were made at 25° C. 1 ml. of freshly prepared 0·1 M-sodium bicarbonate solution was placed in the side-arm. 1 ml. of 0·1 M-phosphate buffer was placed in the main body of the flask. The pH of the buffer was 6·80 in the preliminary experiments and 6·98 in the final experiments. To the buffer 0·2 ml. of glass-distilled water was added for measuring the rate

of the uncatalysed reaction, while 0·2 ml. of tissue extract or diluted blood was added to determine carbonic anhydrase activity. After equilibration for 10 min the contents of the flask were rapidly mixed and readings taken every 60 sec for 10 min, and at 15 and 20 min after mixing. In calculating the volumes of carbon dioxide evolved, allowance was made for that retained in solution by adopting a value for α_{CO_2} as described by Umbreit, Burris & Stauffer (1949), using the value found by Shedlovsky & MacInnes (1935) for the pK of carbonic acid at 25° C. Repeated determinations were made of the carbonic anhydrase activity of the diluted blood and of the extract from ciliary body and iris, together with determinations of the rate of the uncatalysed reaction.

Estimations of carbonic anhydrase by an indicator method

The method employed was a modification of that described by Roughton & Booth (1946). A barbitone buffer, pH 8·4 as measured by a Cambridge pH meter, was prepared by dissolving 4·05 g barbitone and 15·12 g sodium barbitone in 1 l. of glass-distilled water. To 3 ml. of barbitone buffer were added 2 ml. of glass-distilled water, 0·3 ml. of tissue extract or diluted blood and six drops of 0·04% aqueous solution of bromothymol blue in a stoppered weighing bottle. In the uncatalysed reaction 0·3 ml. of glass-distilled water was used in place of tissue extract or diluted blood. The weighing bottles were kept in ice-cold water for 1 hr. 5 ml. of ice-cold glass-distilled water saturated with carbon dioxide were added rapidly from an all-glass syringe and the time taken for the mixture to reach pH 6·3 as shown by its colour matching that of a standard prepared by adding six drops of bromothymol blue to 10 ml. phosphate buffer (pH 6·3) in a weighing bottle. Repeated determinations of the rate of the uncatalysed reaction and of the activity of lens extract, ciliary body extract and its equivalent blood dilution and of 1:50, 1:100, 1:1000, 1:5000 and 1:10,000 blood dilutions were made successively.

Determination of blood content of extracts from ciliary body and iris

Ashby & Chan (1943) used the benzidine reaction to estimate the blood content of tissues by direct visual comparison of the intensity of blue colour developed prior to the determination of the carbonic anhydrase content. It was felt that this method could be made more reliable if photoelectric measurements were possible.

Albino rabbits were used since extracts prepared from the ciliary body and irides of pigmented animals had a brownish tint which interfered with photoelectric measurements. The concentrations and amounts of the reagents proposed by Ashby & Chan were found to be unsuitable because a precipitate was often formed and the development of the blue colour was too rapid. The reagents found most satisfactory were: (*a*) a benzidine solution freshly prepared by dissolving 0·5 g in 4·5 ml. glacial acetic acid at 50° C, cooling, then adding 20 ml. glass-distilled water and filtering before use; and (*b*) a 3% solution of hydrogen peroxide.

To 0·5 ml. of diluted blood or tissue extract were added 1·4 ml. benzidine solution and 4·7 ml. distilled water. 0·4 ml. hydrogen peroxide solution was added quickly, a stop-watch being started at this moment. The resulting blue solution was transferred to a glass cell and the optical density measured at exactly 60, 120 and 180 sec after addition of hydrogen peroxide, in a 'Spekker' photoelectric absorptiometer, following the procedure described by Delory (1949). Ilford 608 spectrum filters were used. The optical density was found to bear a linear relationship to the amount of blood present for dilutions of blood between 1:100,000 and 1:4000.

In a separate series of experiments it was found that the intensity of blue colour developed by a given blood dilution was reduced approximately two or three-fold if ciliary body extract had been added previously to the diluted blood, but there was still a linear relationship between optical density and amount of blood present. Therefore, in the manometric determinations of carbonic anhydrase, the extract was compared with a blood solution five times more concentrated than that which yielded a blue solution with the same optical density as that produced by the extract when treated in the same way with benzidine and hydrogen peroxide. In this way underestimation of the blood content of the extract was certainly avoided.

A larger volume of ciliary body extract was available in those experiments in which carbonic

anhydrase was estimated by the indicator method, so that in the later experiments a modified procedure was adopted for determining the blood content. Known amounts of blood were added to 0·5 ml. of extract and the photoelectric measurements conducted as before. A graph (see Fig. 3) was then constructed from which the blood content of the extract could be deduced thus obviating the difficulty introduced by the inhibitory action of the extract on the development of the blue colour. The blood content of the extract was assessed, not only from the optical densities measured at 60 sec, but also from those at 120 and 180 sec, the largest value of the three being accepted.

Storage and use of extracts

All extracts and blood dilutions were stored in a refrigerator at 0° C in stoppered tubes. The duration of storage before use varied from 24 to 72 hr in the manometric determinations but did not exceed 24 hr when the indicator method was used. There was no evidence of decay in carbonic anhydrase activity. Individual determinations of activity in the equivalent blood dilutions were always carried out immediately after a determination of activity in ciliary body extract.

RESULTS

Manometric method

Extracts from ciliary body and iris. Extracts from six rabbits were tested for carbonic anhydrase activity.

Three extracts were tested at pH 6·80 and the carbonic anhydrase activity was found to be greater than could be explained by the amount of blood present. However, the rate of evolution of carbon dioxide was too rapid to permit readings for more than 4–5 min.

TABLE 2. Manometric determinations of carbonic anhydrase activities at 25° C of extracts from ciliary body and iris and of equivalent dilutions of blood

	Weight of ciliary body and iris (mg)	Velocity constants Mean ± S.E. (no. of determinations)		
		Uncatalysed reaction	1:20,000 dilution of blood added	Extract added
Rabbit 1052	57	0·0811 ± 0·0033 (10)	0·0801 ± 0·0025 (10)	0·1107 ± 0·0023 (7)
Rabbit 1098	105	0·0817 ± 0·0037 (10)	0·0854 ± 0·0045 (10)	0·1025 ± 0·0013 (9)
Rabbit 1186	65	0·0766 ± 0·0035 (10)	0·0800 ± 0·0058 (10)	0·1066 ± 0·0026 (10)

For the remaining three extracts a phosphate buffer pH 6·98 was used. When benzidine and hydrogen peroxide were added, each extract yielded a blue solution with the same optical density as that obtained by adding the same reagents to a 1:100,000 dilution of blood from the corresponding animal. Accordingly, for the reasons given above, the carbonic anhydrase activity of each extract was compared with that of a 1:20,000 dilution of blood.

The results were expressed as constants related to the velocity of the reaction and are given in Table 2. The constants were calculated as suggested by Guggenheim (1926), a procedure which has been applied to determinations of carbonic anhydrase activity by Mitchell, Pozzani & Fessenden (1945) and

by Altschule & Lewis (1949). The volumes of carbon dioxide evolved during the first 9 min of the reaction were paired for 5 min intervals, the logarithms of the volume changes were plotted against time and the linear relationship confirmed. The constant for each determination was found by calculating the regression of log (volume change) on time. In Table 2, the constants for the reaction with extract added are significantly greater than those when the equivalent dilution of blood was added ($0{\cdot}1 > P > 0$). The results for one of these animals are illustrated in Fig. 1, showing that the final volume of carbon dioxide evolved was not altered by the addition of diluted blood or extract. Single determinations on heated extracts gave rates falling within the range of the uncatalysed reaction.

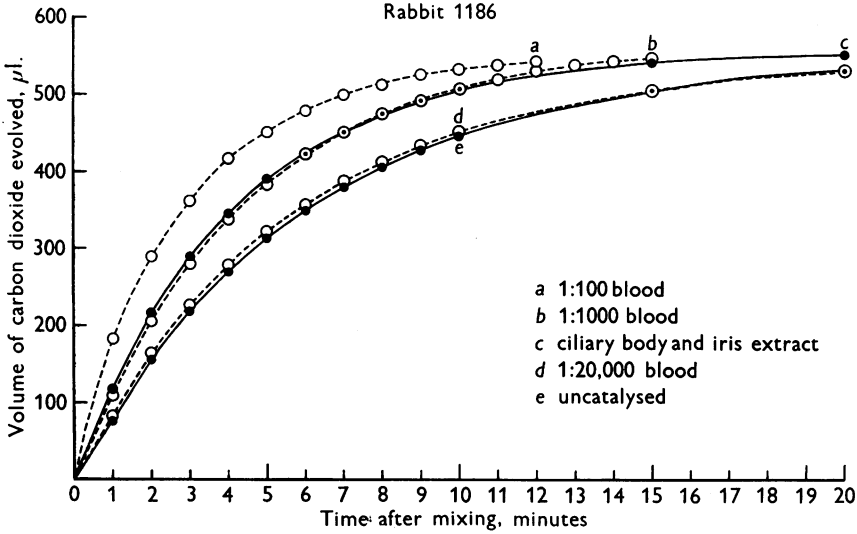

Fig. 1. Carbonic anhydrase activity of extract from ciliary body and iris determined by manometric method. *c*, *d* and *e* are each constructed from the means of ten determinations; *a* and *b* are results of single determinations.

Lens extracts. The results for extracts prepared separately from the lenses of six animals are shown in Fig. 2, from which it is seen that addition of lens extract approximately doubled the initial rate of carbon dioxide evolution. With heated lens extracts, the rate of evolution equalled or was very little greater than that of the uncatalysed reaction.

Indicator method

Extracts from ciliary body and iris. Extracts from six further rabbits were tested by this method.

The first three extracts had a greater carbonic anhydrase activity than could be attributed to their blood content, the latter being determined by the same method as for those extracts tested by the manometric method.

For the remaining three extracts, the blood content was determined by the modified procedure. An example is illustrated in Fig. 3, which shows that this extract contained no more than 50 p.p.m. of blood, no matter whether the 60, 120 or 180 sec readings were considered. The pH change took place more rapidly when extract was added than when an amount of blood equal to that in the extract was used, and the difference between the rates was significant. The rate with heated extracts was not significantly different from that of the uncatalysed reaction. These results are summarized in Table 3.

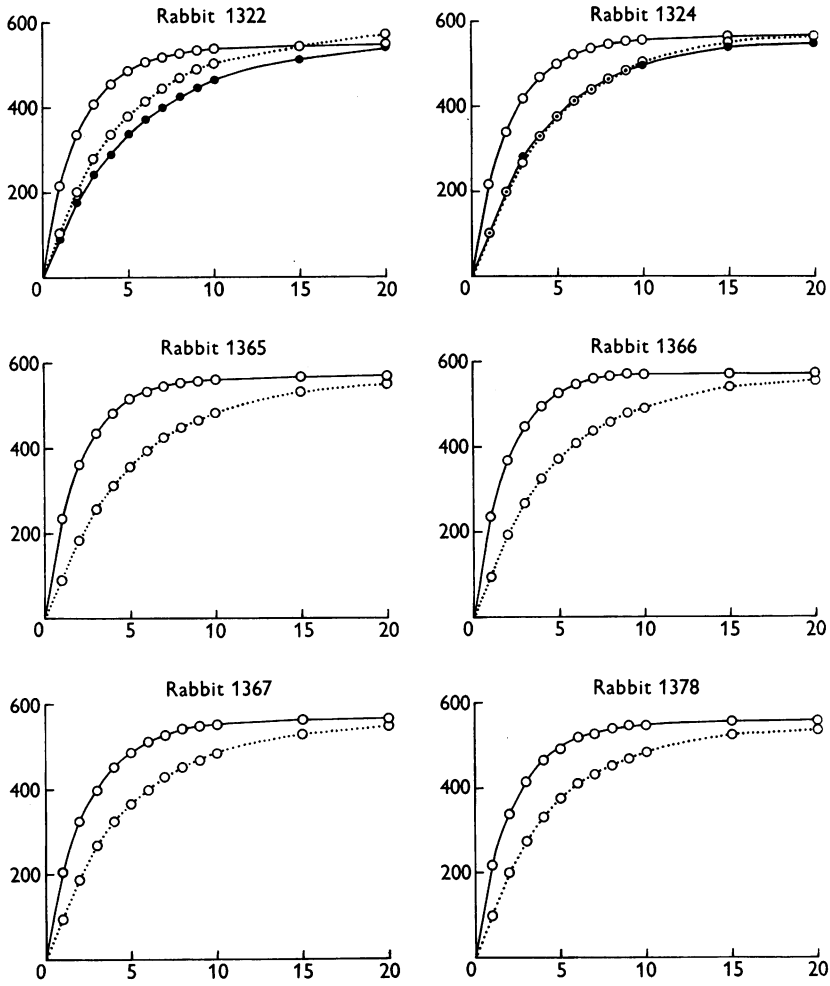

Fig. 2. Carbonic anhydrase activity of lens extracts. Ordinates: μl. CO_2 evolved. Abscissae: time (min) after mixing. Each curve shows the results of a single determination: O—O, lens extract; O···O, heated lens extract; ●—●, uncatalysed reaction.

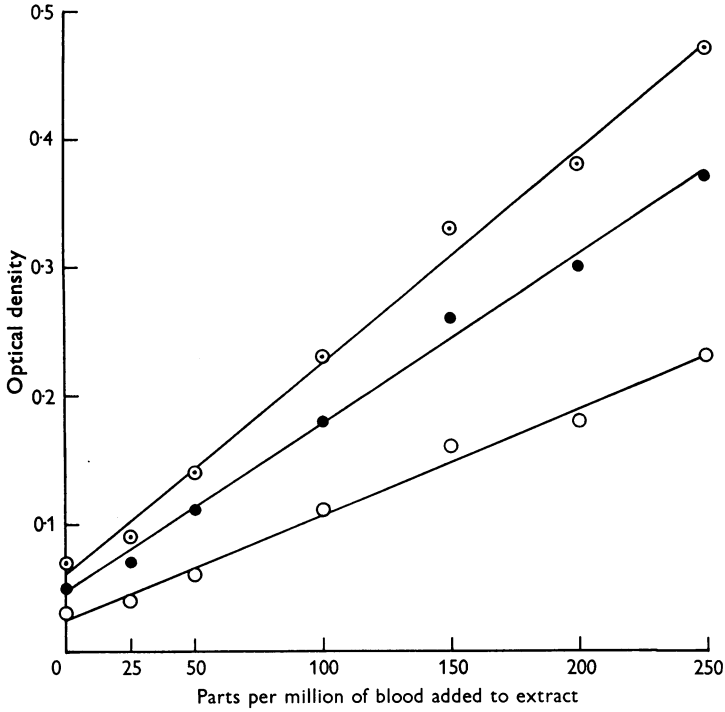

Fig. 3. Determination of blood content of extract from ciliary body and iris. ○, readings taken 60 sec after adding H_2O_2; ●, readings taken 120 sec after adding H_2O_2; ⊙, readings taken 180 sec after adding H_2O_2. The calculated regression lines have been drawn.

TABLE 3. Determination of carbonic anhydrase activity by an indicator method at 0° C

	Time (sec) for pH change from 8·4 to 6·3 Mean ± S.E. (no. of determinations)		
	Rabbit 1279	Rabbit 1474	Rabbit 1243
Uncatalysed	157·5±2·8 (10)	143·3±2·3 (10)	178·9±1·5 (10)
Blood diluted			
1:40,000	—	—	*181·7±1·8 (10)
1:20,000	*159·3±2·5 (10)	—	—
1:10,000	150·6±3·1 (5)	*141·2±2·5 (10)	185·7±1·8 (5)
1:5000	144·8±2·9 (5)	134·8±5·9 (5)	168·1±1·5 (5)
1:1000	99·9±2·1 (5)	102·7±2·7 (5)	133·6±2·4 (5)
1:100	19·7±0·43 (5)	22·1±0·45 (5)	25·0±0·24 (5)
1:50	9·8±0·14 (5)	11·5±0·21 (5)	12·7±0·24 (5)
Extract from ciliary body and iris	144·4±1·8 (10)†	125·7±3·2 (10)†	157·3±2·5 (10)†
Heated extract	161·0±0·53 (3)	158·2±4·3 (4)	175·2±6·8 (3)
Lens extract	11·4±0·24 (5)	9·7±0·37 (5)	13·5±0·26 (5)
Heated lens extract	139·1±3·4 (3)	149·1±3·5 (4)	160·1±2·0 (3)
Weight of ciliary body and iris	66 mg	95 mg	91 mg
Weight of lenses	795 mg	1010 mg	1053 mg

* The blood content of each extract from ciliary body and iris was equivalent to that blood dilution marked by an asterisk in the appropriate column.

† The times for these extracts are significantly different from (*a*) the times for the equivalent dilutions of blood ($0·01>P>0$ in each case; $t=4·89$, 3·86 and 8·04 respectively), and (*b*) the times for the heated extracts ($0·01>P>0$ in each case; $t=4·59$, 5·69 and 3·11 respectively).

Lens extracts. Table 3 shows that the addition of lens extract greatly accelerated the reaction, and that this effect was reduced markedly if the extract had been heated previously. Nevertheless, with two of the heated extracts (from rabbits 1279 and 1243) the reaction was faster than the uncatalysed reaction.

DISCUSSION

The results obtained by both the manometric and the indicator methods demonstrate that a greater amount of carbonic anhydrase was present in extracts prepared from the ciliary body and iris of albino rabbits than could be explained by the blood content of the extracts. Table 3 shows that the extracts had carbonic anhydrase activities approximately equal to blood diluted 1:5000 for rabbit 1279, 1:4000 for rabbit 1474 and 1:3000 for rabbit 1243. Allowing for the amounts of blood present, the extracts were approximately equivalent in carbonic anhydrase activity to blood diluted 3:20,000, 3:20,000 and 6:20,000 times respectively. From the amounts of tissue used and the final volumes of the extract it can be calculated that the concentration of carbonic anhydrase in the ciliary body and iris of each of the three animals was about 1/45, 1/65 and 1/30 respectively of the concentration in whole blood. This is considerably lower than the amount claimed by Wistrand (1951), who stated that the concentration in the ciliary body and iris was about 1/7 of that in the erythrocytes, but he did not specify whether the animals used were pigmented or albinos. Bakker (1939) found more carbonic anhydrase in the lens of an albino rat than was usual in pigmented rats, and Leiner (1940) obtained a similar result for the choroid and retina of an albino rabbit. These results suggest that the carbonic anhydrase contents of the tissues may differ between albino and pigmented animals.

The lens extracts from rabbits 1279, 1474 and 1243 all had approximately the same carbonic anhydrase activity as 1:50 dilutions of blood. Taking into account the weights of the lenses and the final volumes of the extracts, it may be calculated that the lenses possessed 1/4, 1/5 and 1/5 respectively of the carbonic anhydrase activity of whole blood. The finding that some heated lens extracts cause a small acceleration of the reaction may be due perhaps, as Bakker (1939) suggested, to lactic acid formation in the lens.

By comparing the carbonic anhydrase activities, expressed as fractions of the activity of whole blood, of lens extracts and extracts from ciliary body and iris, it is concluded that the concentration of carbonic anhydrase is 6 to 13 times greater in the lens than in the ciliary body and iris, assuming a uniform distribution of the enzyme in both tissues. Nothing appears to be known about the localization of the enzyme in these tissues, and it may well be that a high proportion of the carbonic anhydrase of the ciliary body is localized in the ciliary epithelium which would therefore possess a concentration equal to, or perhaps greater than, that of the lens.

SUMMARY

1. The carbonic anhydrase in extracts prepared from the lens and from the ciliary body and iris of albino rabbits has been estimated manometrically and by an indicator method.

2. A method is described by which the blood content of extracts from the ciliary body and iris was determined by using the benzidine reaction and measuring the resulting blue colour in a photoelectric absorptiometer.

3. Extracts from the ciliary body and iris contained more carbonic anhydrase than could be accounted for by the amount of blood they contained.

4. The lens extracts had a high carbonic anhydrase activity.

5. The carbonic anhydrase content of the ciliary body and iris appeared to be between 1/30 and 1/65 of that of whole blood, while the amount in the lens was between 1/4 and 1/5 of that in blood. Therefore, in equal weights of the two tissues, there was 6 to 13 times as much carbonic anhydrase in the lens as in the ciliary body and iris.

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