Histochemie 11, 112—128 (1967)

Histochemical Demonstration of Carbonic Anhydrase Activity*

HOLGER P. J. HANSSON
Departments of Pharmacology and Dermatology, University of Uppsala, Uppsala, Sweden

Received June 21, 1967

Summary. Freeze-dried frozen sections are floated on the surface of the freshly prepared incubation mixture ($CoSO_4$ 1.75×10^{-3} *M*, H_2SO_4 5.3×10^{-2} *M*, $NaHCO_3$ 1.57×10^{-2} *M* and KH_2PO_4 1.17 to 11.7×10^{-3} *M*; demonstration of weak activity requires high phosphate). A compound containing cobalt and phosphorous precipitates at carbonic anhydrase sites and is converted to CoS. Adequate staining requires only 2—10 minutes of incubation. Actazolamide inhibits the staining reaction in specific concentrations. Actazolamide *in vivo*, 20 mg/kg *i.v.* to mice 30 minutes before sacrifice also inhibited the staining. The proportion phosphorous in the specific precipitate increases with KH_2PO_4 of the medium (shown by the addition of ^{60}Co and ^{32}P). An explanation of the reaction mechanism is given, based on the catalyzed loss of CO_2 in the surface layer. The inclusion of phosphate in the medium makes this modification of HÄUSLER's method so sensitive that it shows carbonic anhydrase activity in for instance stratum spinosum of the skin.

Introduction

Highly specific and non-toxic carbonic anhydrase inhibitors exist and are used not only in physiological investigations but also in medical practice. Therefore it is of considerable importance to identify the cells which contain the enzyme. Attempts in this direction have been made using cytochemical studies after microdissections (DAVENPORT, 1939; GIACOBINI, 1962); direct fluorescent antibody technique (HANSSON, 1965) and enzyme histochemistry.

HÄUSLER (1958) described a histochemical method which was slightly modified by WALDEYER and HÄUSLER (1959). In this method sections are kept floating on the incubation mixture. The reaction is inhibited by acetazolamide and has been accepted as specific by the majority of workers.

In the method of WALDEYER and HÄUSLER (1959) the incubation medium is freshly prepared by mixing an acid solution of $CoSO_4 + H_2SO_4 + Na_2SO_4$ with a solution of $NaHCO_3$. Immediately after mixing when pH is abount 7.3, sections are floated on the surface of the mixture. During the incubation (90—120 min) carbon dioxide leaves the solution and a reaction product which includes cobalt precipitates in the sections. The cobalt is made visible by $(NH_4)_2S$. The mode of action of the method has not been clarified but an attempt at explanation is made in the present paper.

KORHONEN and KORHONEN (1965a) have modified the concentrations of the WALDEYER and HÄUSLER method and the fixation technique. The modification allows somewhat shorter incubation times, but 15—20 minutes are still needed.

The long incubation times in the HÄUSLER method and its congeners are a serious drawback, since diffusion of the enzyme or the reaction product may take place.

* This investigation was supported by grants from the Medical Faculty, University of Uppsala and from the U.S. National Institutes of Health (Grant NB 3060 to E. BÁRÁNY).

In unpublished experiments, the present author has found 50% of the total enzymatic activity to leave unfixed 20 μ sections on glass slides submerged for 5 minutes. The same loss of enzyme activity was found despite prior treatment of the sections with acetone, ethanol or formaldehyde at different pH and concentrations of NaCl. Diffusion of the enzyme out of the sections could therefore be a serious source of error and *a radical shortening of incubation time* is highly desirable. This has been achieved in the method described below.

Throughout the paper, the fact that acetazolamide in low concentrations inhibits a reaction is taken as proof that the reaction is caused by carbonic anhydrase (CA) activity. This point of specificity will also be touched upon in the discussion.

Material and Methods

Preparation of Specimen. Tissues were obtained from human skin after punch biopsy and stomach during surgery; from monkeys (*Macaca irus* and *Cercopithecus aethiops*) killed by an overdose of sodium pentothal and from exsanguinated mice and toads.

The tissues were cut into small blocks, one dimension not more than 2—3 mm. The pieces were put singly into very small bags of thin polyethylene, the air gently squeezed out as completely as possible and the bag with tissue put into solid CO_2-acetone. The whole procedure took about 1 minute. In other experiments the tissue samples were immediately fixed and then frozen as described. Sections were cut (10—20 μ) on a Pearse freezing microtome and then dried under reduced pressure at —75° C in the presence of P_2O_5. These sections kept their CA activity unaltered for at least 5 months.

Thinner sections sometimes desired for histological reasons tend to disintegrate when floated on the incubation mixture. Such sections (5—7 μ) were therefore thawed on a Millipore filter (TH, 25 μ thick, pore size 0.45 μ) and subsequently handled on the filter.

Composition of Media. All solutions used are kept at room temperature (22° C) where not otherwise stated. The incubation medium of WALDEYER and HÄUSLER (1959) has been altered by elimination of Na_2SO_4 and addition of KH_2PO_4. The two components are made up according to the following (enough for one 9.5 cm Petri dish): To solution I containing 1 ml 0.1 *M* $CoSO_4$, 6 ml 0.5 *M* H_2SO_4, 1—10 ml 1/15 *M* KH_2PO_4 and distilled water to 17 ml, solution II containing 0.75 g $NaHCO_3$ in 40 ml distilled water (freshly prepared) was added, providing a final molar concentration of 1.75×10^{-3} $CoSO_4$, 5.3×10^{-2} H_2SO_4, 1.17—11.7×10^{-3} KH_2PO_4 and 1.57×10^{-2} $NaHCO_3$, (without consideration of the rapid formation of CO_2 at the pH 5.8 of the solution). In the controls of the specificity of the staining reaction sodium acetazolamide was included in solution II.

Blackening Solution. The cobalt present in the sections was visualized by a freshly prepared solution of 0.5% $(NH_4)_2S$ in distilled water. This was filtered through a filterpaper where CoS had been deposited. Some grains of CoS were added to the filtrate for saturation before use.

Total Procedure. The following procedure was employed in the present experiments:

1. Pour solution II over solution I in a Petri dish (diameter 9.5 cm) at room temperature (22° C) and float sections on the surface of the mixture. Make sure that the sections do not dip under the surface of the medium. Incubate for 2 minutes or more.

2. Float sections onto the rinsing solution for 1—3 minutes. It does not matter if they dip under the surface.

3. Float sections on the blackening solution for 3 minutes. It does not matter if they dip under the surface.

4. Rinse by floating the sections for 1 minute in each of three successive saline dishes.

5. Dry sections onto slides. To assure good adhesion during counterstaining one drop of Carnoy's fixation solution (glacial acetic acid 1 part, absolute ethanol 6 parts, chloroform 3 parts) may be placed over the section and allowed to dry for 5 minutes at 37° C.

6. If desired, counterstain with e.g., nuclear fast red or haematoxyline and eosin or toluidine blue.

7. Mount sections with glycerol jelly or dehydrate sections through graded concentrations of ethanol, xylene and mount with Canada balsam.

Sites of CA reaction are stained black (CoS) and there is no visible staining in the inhibited control sections.

Determination of the Deposition of Cobalt and Phosphate in the Sections. To solution I was added enough ^{32}P as phosphate to give about 10^{10} cpm/mol P and enough ^{60}Co as sulfate to give about 2×10^{10} cpm/mol Co. A suitable number of sections (five) were selected at random from a large number kept in the dry state in a Petri dish. They were incubated and subsequently washed as described under steps 1 and 2. They were placed together on an aluminium planchet and dried at 37° C. The sum of the radioactivity of the five sections was determined using a Tracerlab SC-18 Superscaler with a TGC-1 end-window Geiger-tube (window 2.8 mg/cm^2). The activity was first counted immediately after the incubation and then 75 or 88 days later when most of the ^{32}P but very little of the ^{60}Co had decayed. Accurate determination of cpm/mol in the solutions were also made, using the same counter and planchets. Thus, two equations for the total activity can be set and by a simple calculation one obtained the amount of cobalt and phosphorous present in the five sections.

Inhibitors. The inhibitors tested included: sodium acetazolamide (Diamox® Sodium, American Cyanamid Company) and chlorothiazide (Chlotride®, Merck Sharp & Dohme). As controls the N^5-t-butyl derivate of acetazolamide, CL 13850 (American Cyanamid Company) said to be devoid of CA inhibitory activity (MAREN, 1956) replaced sodium acetazolamide. The inhibitors were included in the incubation mixture.

Results

1. Fixation. In experiments on toad kidney, fresh unfixed freeze-dried sections were used as well as such sections from tissues fixed for 3 hours in 6.25% glutaraldehyde; for 5 hours in 6.25% α-hydroxyadipaldehyde buffered to pH 7.25 with 0.15 *M* cacodylate or for 5 hours in 6.25% α-hydroxyadipaldehyde unbuffered in 0.88 *M* sucrose. The toad kidney also was perfused *in vivo* with 6.25% glutaraldehyde buffered to pH 7.3 with 0.15 *M* cacodylate and the excised tissue slices subsequently fixed for 20 hours in the same mixture. In these experiments no differences in the distribution of staining and only slight differences in the intensity (amount of staining per unit tissue) were seen. However, a nuclear staining often was noticed after fixation. The tissues also were found to retain their histochemical stainability when stored after the fixation in 0.88 *M* sucrose solution which contained 7% of polyvinylpyrrolidone as recommended by KORHONEN and KORHONEN (1965b).

Tissue slices from human skin, however, showed not only fewer sites of staining but also a lessened intensity when fixed in glutaraldehyde buffered to pH 7.2. This might be due to a selective loss of CA activity. Biochemical measurements with the method of MAREN, ASH and BAILY (1954) have shown that one of the human isoenzymes (HCA C) loses more than 90% of its enzymatic activity after treatment for one hour with 2.5% glutaraldehyde, but the other one tested (HCA B) is unaffected (HANSSON, to be published).

No experiments were performed on paraffin embedded tissues since freeze-dried sections passed before incubation through ethanol (40, 80, 96 and 99.5%) to xylene and then back again showed fewer reactive sites and also needed markedly longer incubation times for equal staining.

In the following, unfixed freeze-dried sections were used, where not otherwise stated.

2. Cobalt. When cobalt was excluded from the incubation medium containing 1 ml of 1/15 *M* KH_2PO_4 there was no staining of the sections. The reaction was dependent on the final cobalt concentration of the solution. Thus at 3.5×10^{-4} *M*

very few sites were stained within 17 minutes. At $8.75 \times 10^{-3} M$ precipitation rapidly appeared on the surface of the incubation medium leading to diffuse black staining in the sections also in the presence of acetazolamide. Under the conditions chosen a final cobalt concentration of $1.75 \times 10^{-3} M$ was found suitable.

3. Visualisation of the Reaction Sites. Before the cobalt was developed with sulfide the sections were rinsed. This step was found critical. Short treatment (1—3 min) and the use of physiological saline buffered with phosphate (6.7×10^{-4}, pH 5.9) was essential. The reason for the addition of phosphate will presently be apparent. Optimal black staining of the sections was obtained when they were treated for 3 minutes with 0.5—2% $(NH_4)_2S$.

4. Deposition of ^{60}Co and ^{32}P in the Sections. Sections, 20 μ, from the glandular part of mouse stomach were incubated in the presence of ^{60}Co and ^{32}P (see Methods). After incubation for various periods the sum of radioactivity was measured in batches of sections for each time. One additional section was treated for light microscopy. Fig. 1 shows a typical experiment. The sections were incubated for 5, 15 and 25 minutes with 1 ml $1/15 M$ KH_2PO_4. The figures are corrected for the unspecific uptake of cobalt by the sections (see below). The deposition of cobalt and phosphorous increased with the time of incubation and to about the same extent indicating that a precipitate of approximately constant composition is formed. Also the number of stained sites and the intensity of staining increased with time, from very little at 5 minutes to extensive deposition at 25 minutes. Sections incubated with acetazolamide, $2 \times 10^{-5} M$ in the medium did not show any deposition of phosphorus within 25 minutes, and the staining reaction was totally inhibited. Thus, if sections were stained the reaction had given a precipitate which contained cobalt as well as phosphorous. The deposition was caused by the activity of CA, since it was inhibited by acetazolamide. (More about the specificity below.) The absence of phosphorous in the inhibited sections made it clear that neither catalyzed nor uncatalyzed precipitation had occured. The cobalt found must therefore be due to the binding of cobalt by the tissue. It was evidently so diffusely distributed that it did not stain visibly with sulphide.

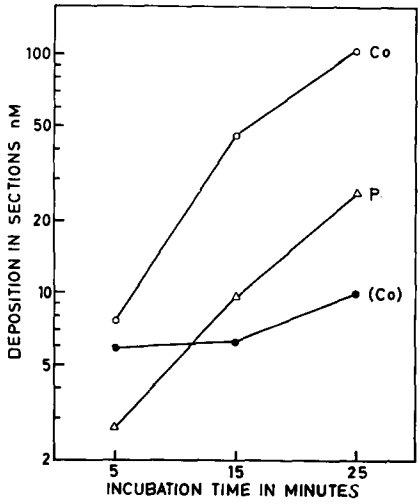

Fig. 1. Effect of incubation time on amounts of cobalt and phosphorous deposited in five (20 μ) sections from the glandular part of mouse stomach. One ml $1/15 M$ KH_2PO_4/57 ml medium. The deposition of cobalt, Co, is corrected for the amount, *(Co)* present in inhibited sections ($2 \times 10^{-5} M$ sodium acetazolamide in the medium). The inhibited sections contained no deposited phosphorous. The *ordinates* in nanogram atoms

The deposition of cobalt and phosphorous in the sections also has been measured with CA inhibitors included in the incubation medium. Sections from the same mouse stomach were used and incubated for 7 and 25 minutes. The experiment

was performed in the same manner as that above. The total amounts of cobalt and phosphorous in the five sections incubated are shown in Fig. 2. Without inhibitor in the medium the sections were stained and more so after the incubation for 25 minutes. Also the amounts of cobalt and phosphorous increased. When acetazolamide, $2 \times 10^{-5} M$, was added, a low amount of cobalt and very little,

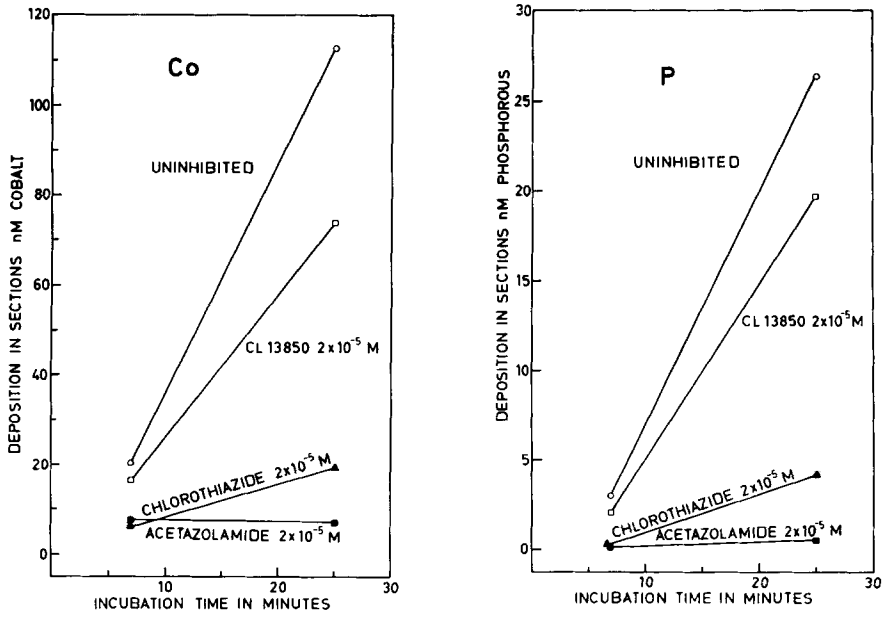

Fig. 2. The *in vitro* effect of inhibitors on the amounts of cobalt and phosphorous deposited in five (20 μ) sections from the glandular part of mouse stomach during incubation. One ml $1/15 M$ KH_2PO_4/57 ml medium. The *ordinates* in nanogram atoms

if any phosphorous was present in the sections. The staining was completely inhibited. With chlorothiazide, $2 \times 10^{-5} M$ in the medium the amounts measured after 7 minutes corresponded to those found with acetazolamide. After 25 minutes, however, increased amounts of cobalt as well as phosphorous could be found but not as much as in the uninhibited sections. Visually there was staining in the 25 minute section but not in the 7 minute ones. The slight inhibition shown by CL 13850, $2 \times 10^{-5} M$ can possibly be due to contamination with acetazolamide (see MAREN, 1956). The batch of CL 13850 also exhibited some slight inhibitory activity against CA activity from human hemolysates (about 18% inhibition by 10^{-4} M) when it was tested *in vitro* with the method of MAREN, ASH and BAILY (1954).

Fig. 3 shows the effect of different concentrations of phosphate. Sections from monkey duodenum and human facial skin were used. The same technique as described was applied and the sum of radioactivity in 5 sections was measured. It is seen that with very low concentration of phosphate mainly cobalt was deposited. Evidently the reaction here proceeds as in the original HÄUSLER method, the precipitate probably being basic carbonate or hydroxide. With increasing concentration of phosphate, very much more cobalt is fixed, but now together with phosphate.

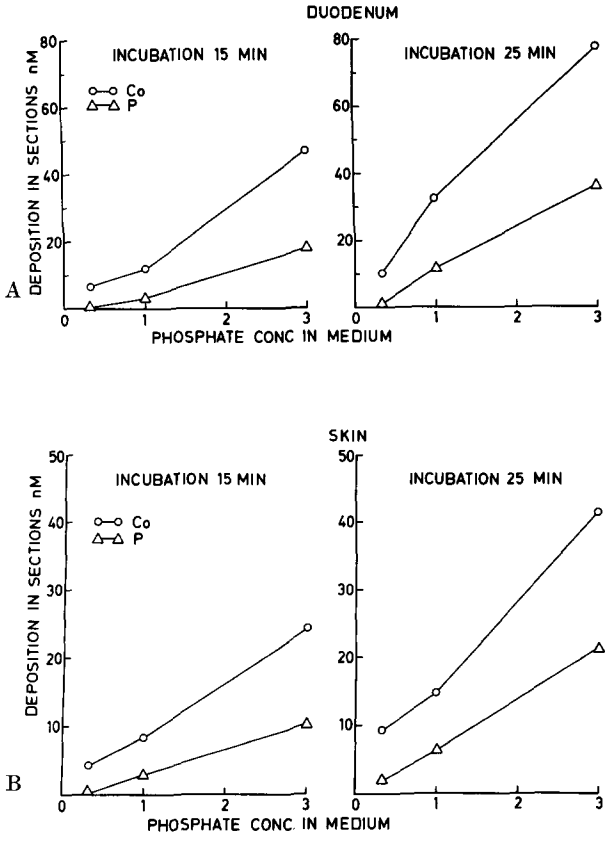

Fig. 3 A and B. Effect of phosphate concentration in ml of 1/15 *M* KH_2PO_4/57 ml medium on the amounts of cobalt and phosphorous deposited in five (20 μ) sections during incubations for 15 and 25 minutes. A, Monkey duodenum. B, Human skin. The *ordinates* in nanogram atoms

Table 1. *Ratio P/Co deposited in sections at different KH_2PO_4 concentrations*

Tissue[a]	KH_2PO_4 1/15 *M*		
	0.33 ml[b]	1.00 ml[b]	3.00 ml[b]
Human skin	0.30[c]	0.33	0.62
Monkey duodenum	0.26	0.41	0.58
Monkey stomach	—	0.33	—
Mouse stomach	—	0.30	—

[a] Five sections, 20 μ. [b] In 57 ml of medium.
[c] Ratio of the difference between deposition in sections incubated for 25 and 15 minutes.

Fig. 4 illustrates the staining reaction in the skin sections after 15 minutes.

Table 1 shows that the composition of the precipitate formed between minutes 15 and 25 depends on the phosphate content of the solution. More phosphate is bound per unit of cobalt if more is available.

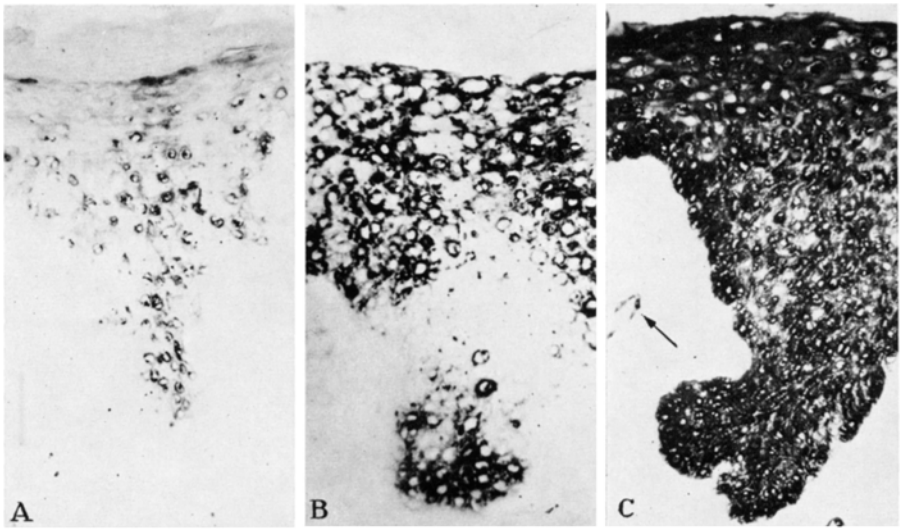

Fig. 4 A—C. Human facial skin (case of light-dermatosis). Comparison between the staining reaction at different phosphate concentrations. Amounts in ml of 1/15 *M* KH_2PO_4/57 ml medium: A. 0.33, B. 1.0, C. 3.0. Incubation for 15 minutes. Same batch of sections as used in Fig. 3B. The arrow indicates reaction in a capillary loop of the dermal papillae. × 128

From Table 2 which is based on the experiments shown in Fig. 2 it is evident that the enzymatic activity state of the sections does not greatly influence the composition of the precipitate formed.

Table 2. *Deposition of P and Co in sections*[a] *and their ratio (P/Co) at different enzyme activities*

Medium[b]	Precipitation nanogram atoms[c]		Ratio
	P	Co	P/Co
No inhibitor	23.2	92.3	0.25
CL 13850 2×10^{-5} *M*	17.6	57.8	0.33
Chlorothiazide 2×10^{-5} *M*	4.0	13.2	0.33

[a] Mouse stomach, sum of five sections (20 μ).
[b] 1 ml 1/15 *M* KH_2PO_4/57 ml.
[c] Differences between sections incubated for 25 and 7 minutes.

5. Staining Experiments with Phosphate-Containing Solutions. Addition of phosphate to the incubation medium of WALDEYER and HÄUSLER (1959) was found radically to alter the conditions of CA staining in the tissue sections. The most striking feature is the markedly shortened incubation time required. This enhanced sensitivity of the method is easiest to notice in sections with a low degree of CA activity. Thus it was found suitable to test the effect of phosphate with sections from human skin. The procedure was as described in the method. Fig. 5 shows a typical experiment. The figures on the time axis indicate which incubation times were actually used. The points in the diagram show combinations of time and phosphate which caused just discernible staining. Without

phosphate in the medium a very faint and diffuse staining reaction was found after about 90 minutes. Addition of phosphate shortened the times of incubation for about the same staining intensity. When 3 ml of 1/15 *M* KH_2PO_4 was included in the incubation mixture first signs of staining reaction were found in these sections after 6 minutes. However, to counteract the diffusion of the enzyme it will sometimes be favourable to choose still shorter incubation times. Elevating the phosphate concentration affords this possibility. Figs. 6A and 6B show the increased staining reaction in human skin sections incubated for 4 and 10 minutes when the incubation mixture contained 10 ml 1/15 *M* KH_2PO_4. The incubation time is also shortened if the temperature is raised. Fig. 6C shows the intense and rapid (5 minutes) staining reaction in sections incubated at 37° C while those incubated at 4° C (Fig. 6D), are stained only at some sites and after prolonged incubation (22 minutes).

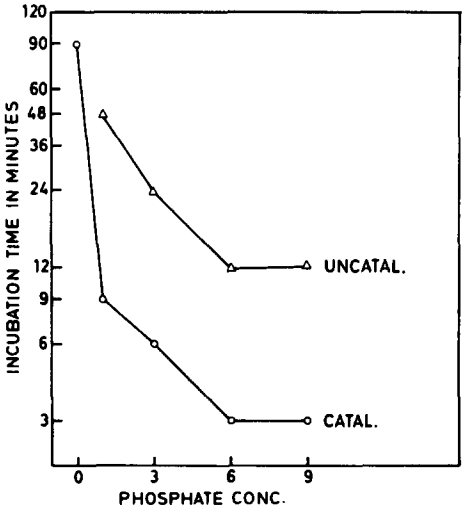

Fig. 5. Incubation time for catalyzed and uncatalyzed first visible staining reaction at different phosphate concentrations. Sections (20 μ) from human skin. Abscissa: ml 1/15 *M* KH_2PO_4/57 ml medium

6. Time Limit for the Uncatalyzed Deposition. The incubation time for the specific deposition of cobalt in the sections is limited upwards by the time needed for the uncatalyzed reaction to give its deposition. The uncatalyzed reaction shows up as a diffuse grayblack staining of the tissue and the appearance of grayblack scales surrounding the edges of the sections. The concentration of phosphate in the medium influences the time of appearance of the uncatalyzed reaction. As shown in Fig. 5 it takes place earlier the higher the concentration of phosphate. The time lag between the first visible specific staining of the sections and the uncatalyzed precipitation is shortened by phosphate. Thus if sites of different enzyme activity are to be compared in a semiquantitative manner it is advisable to use 1—3 ml 1/15 *M* phosphate in the solution and follow the increasing staining reaction at suitable intervals until the uncatalyzed precipitation comes at abount 48—24 minutes. However, the least loss of enzyme is given by higher concentrations of phosphate (6—10 ml 1/15 *M* KH_2PO_4). Then the incubation times are limited to about 12 minutes by the unspecific reaction.

7. Inhibition of Histochemical CA Activity. Inhibition in vitro. To check the specificity of the staining reaction, controls were run with CA inhibitors. For comparison one highly active inhibitor (sodium acetazolamide) and one less active (chlorothiazide) and one control sulfonamide (CL 13850) were employed. In a typical experiment freeze-dried sections of kidney, stomach and skin (cynomolgus) were incubated for 15 minutes with 1 ml 1/15 *M* phosphate in the medium using different inhibitor concentrations. Uninhibited the sections stained heavily. The two inhibitors completely abolished visible staining (acetazolamide at 10^{-6} *M*,

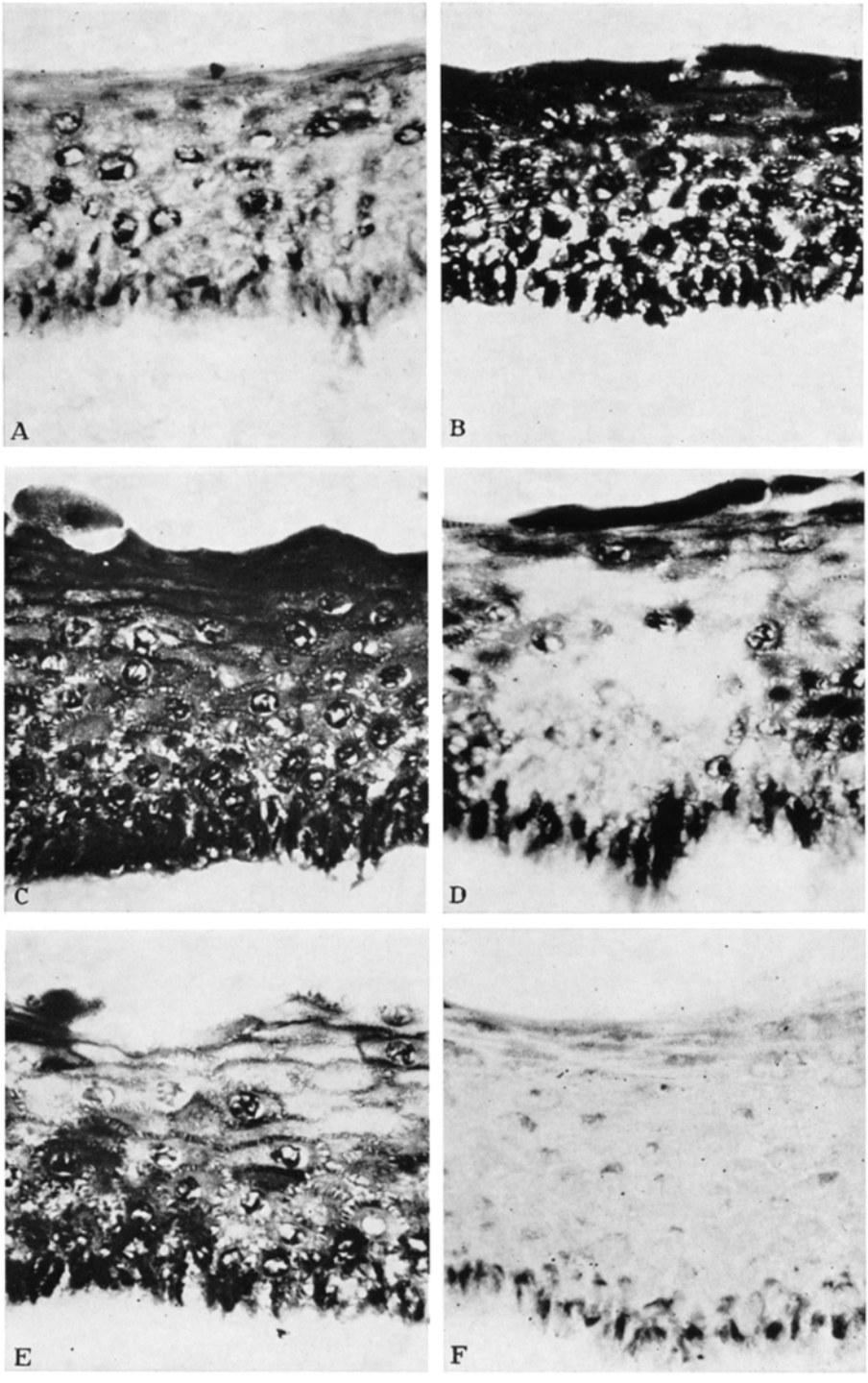

Fig. 6 A—F (for Legends see p. 121)

chlorothiazide at $5 \times 10^{-5} M$). The control substance CL 13850 left the staining reaction unaffected.

Subtotal inhibition of sections from human skin is illustrated in Figs. 6 E and F. In these sections a nuclear staining was found although the sections were unfixed and the duration of incubation was kept shorter than the time necessary for the uncatalyzed precipitation to occur. In other experiments with unfixed freeze-dried sections from the thyroid of cynomolgus a nuclear staining was found only when the reaction mixture contained acetazolamide at a subtotal inhibiting concentration, but was absent at higher concentrations of the inhibitor. When no inhibitor was present only the cytoplasm was stained. An attempt at an explanation will be given in the discussion.

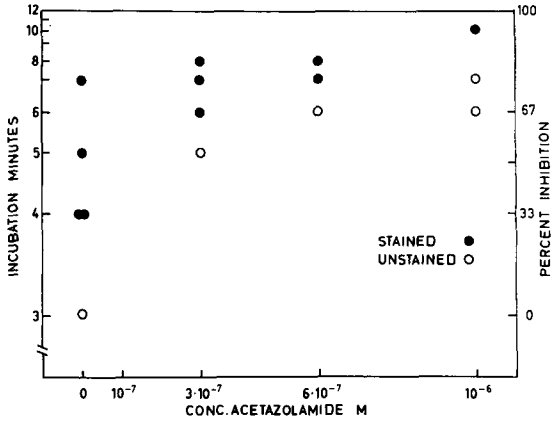

Fig. 7. Dose-response relationship between acetazolamide in the medium and staining reaction in sections from monkey kidney cortex (20 μ) incubated for different times at room temperature. One ml 1/15 *M* KH_2PO_4/57 ml medium. The scale of per cent inhibition assumes that the threshold for visualization is just above 3 minutes when inhibition is zero and that the uncatalyzed staining appears after 12 minutes

The time necessary for visual staining must depend on the enzymatic activity of the structure and the sensitivity of the method. Other things being equal, the inverted time is a rough measure of the rapidity of the reaction. Thus the time for just visible uncatalyzed and for catalyzed staining gives the corresponding reaction velocities v_u and v_e and $v_e - v_u$ is a rough estimate of the enzymatic activity. If this is determined with different concentrations of inhibitor, a dose-response curve for the inhibitor can be constructed. Fig. 7 shows this for sections from monkey kidney cortex incubated at room temperature. It is seen that the time necessary for visualization is roughly 6 minutes with $6 \times 10^{-7} M$ acetazolamide. As the uncatalyzed staining takes 12 minutes and the uninhibited 3 minutes this

Fig. 6 A—F. Human skin from the lower leg (case of panniculitis). Comparison between staining reaction in sections (20 μ) from the epidermis under different conditions. 10 ml 1/15 *M* KH_2PO_4/57 ml medium. A. Incubation for 4 minutes at 22° C, B. 10 minutes at 22° C, C. 5 minutes at 37° C, D. 22 minutes at 4° C, E. 10 minutes at 22° C with $10^{-7} M$ acetazolamide. Partially inhibited staining reaction. F. 10 minutes at 22° C with $10^{-6} M$ acetazolamide. Nearly complete inhibition of the staining reaction. Very light conterstaining with toluidine blue 0.0015 per cent, pH 5.0 in sections A, B, E and F. × 240

means a reduction of the catalytic activity to 1/3. The 50 per cent inhibition point is very roughly at 4.8 minutes which is given by 2—3 $\times 10^{-7} M$ acetazolamide. This is definitely in the "specific" range and far below the concentration of cobalt.

Thus with increasing duration of incubation more acetazolamide is necessary to inhibit the histochemical reaction. The same is true if instead of incubation time the sensitivity of the method is increased by the addition of more phosphate. Table 3 shows such experiments. Here the sensitivity at room temperature increased about twofold as seen from the time for first visible staining when the

Table 3. *Effect of phosphate concentration on time for uninhibited staining and completely inhibiting concentrations of acetazolamide in vitro*

Tissue	First staining without inhibitor (minutes)		Acetazolamide molarity inhibiting all staining during 10 minutes incub.	
	1 ml[a]	10 ml[a]	1 ml[a]	10 ml[a]
Human scalp[b]	4	2	3×10^{-7}	10^{-6}
Human scalp[b]	4	2	3×10^{-7}	6×10^{-7}
Monkey tongue[b]	5	3	3×10^{-7}	6×10^{-7}

[a] 1/15 M KH_2PO_4/57 ml.

[b] The sites of reaction investigated were the epidermis of the skin and the mucosa of the tongue.

amount of phosphate was raised from 1 to 10 ml 1/15 M KH_2PO_4 in the medium. With incubation for 10 minutes the minimal concentrations of acetazolamide which gave total inhibition of staining had to be increased 2—3 times.

Inhibition in vivo. Administration of CA inhibitors may give tissue concentrations comparable with those necessary for inhibition of the histochemical staining reaction. One would expect such treatment alone to give microscopically visible inhibitory effects. This was investigated using kidney and stomach from mice. Sodium Diamox® (parenteral) in physiological saline (20 mg/kg) or the same volume of solvent was injected *i.v.* 30 minutes before the mice were killed by decapitation. Freeze-dried sections from unfixed tissue or tissue fixed in α-hydroxyadipaldehyde were incubated for 10 minutes with 1 ml 1/15 M KH_2PO_4 in the standard medium without inhibitor. There was a staining reaction in the controls (Fig. 8A) but none in the sections taken from treated animals (Fig. 8B). After incubation for 15 minutes also the latter showed staining but not at so many sites as the controls. With the HÄUSLER method MUSTAKALLIO, RAEKALLIO and RAEKALLIO (1960) found a slight to moderate inhibition of the staining reaction in the rat kidney by *in vivo* treatment but only after acetazolamide 1000 mg/kg subcutaneously. It seems therefore that good results of *in vivo* inhibition require short incubations so that the inhibitor is not lost to the medium.

8. Control Experiments. a) Sections from tissues where the enzymatic activity was destroyed by boiling were unreactive.

b) Already HÄUSLER (1958) has shown that elimination of the CO_2-gradient between the incubation mixture and the air, by CO_2 blown over the medium during

the incubation, resulted in complete absence of visible staining in the sections. This observation has been confirmed. The loss of CO_2 from the mixture is thus essential for the formation of the reaction product. The experiment also shows that binding of cobalt to tissue proteins is of no importance at pH 5.8, that of the bulk of the fluid when CO_2 loss is prevented.

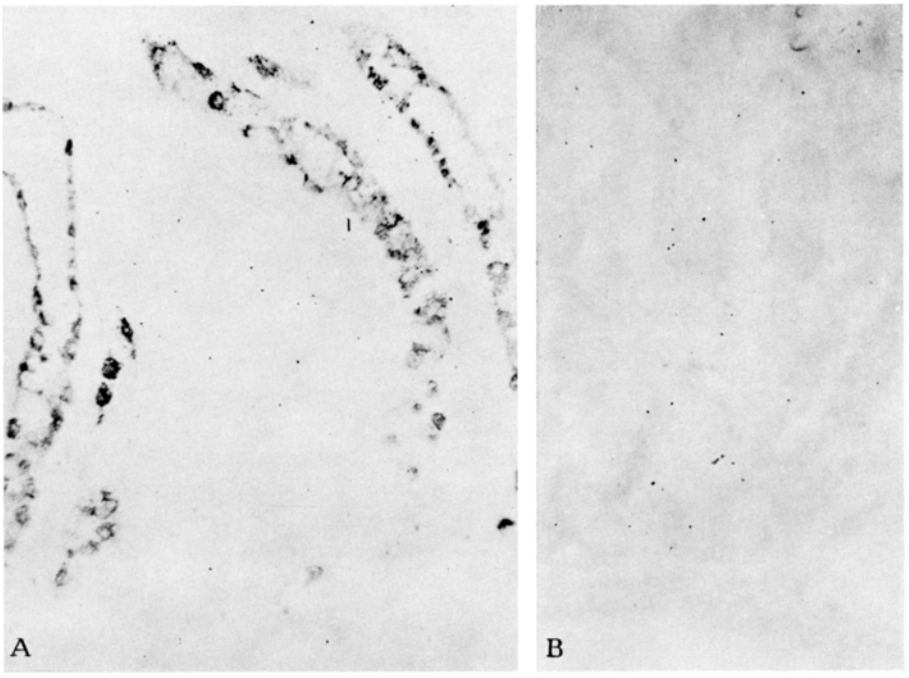

Fig. 8A and B. Gastric glands of mouse. The effect of *in vivo* inhibition on the staining reaction. One ml 1/15 *M* KH_2PO_4/57 ml medium. Sections (20 μ) incubated for 10 minutes. Counterstaining with nuclear fast red. A. Uninhibited control. B. Acetazolamide, 20 mg/kg *i.v.* to mice, 30 minutes before sacrifice. The staining reaction was completely inhibited with this time of incubation. × 128

c) During an ordinary experiment, when CO_2 leaves the incubation medium the pH rises, and first in the surface layer. When equilibrium is established between the partial pressure of CO_2 in the surrounding air and a stirred incubation mixture its pH is 8.5—8.7. Before equilibrium is reached, a lower pH will prevail at the surface but there will be a continuous rise toward the equilibrium value. At one stage, a precipitate forms at the surface, even if no section is present, as already observed by HÄUSLER (1958). If a section is present, unspecific precipitation in the tissue begins around this stage of the process. With the incubation medium as described, precipitate accumulates only very slowly in heavily inhibited sections and no preferential deposition is seen. In order better to see where cobalt precipitates when CA can not be responsible for the deposition, the following experiment was made: The edge of a piece of Millipore filter carrying a few sections was covered with paraffin jelly, forming a little through. This was floated on a cobalt mixture with the sections upwards until wet through. The mixture contained 10 ml 0.1 *M* $CoSO_4$, 3.75 ml 0.8 *M* Na_2SO_4, 10 ml 1/15 *M* KH_2PO_4 and

32.25 ml distilled water, but no $NaHCO_3$. A drop of 0.05 *M* barbital buffer, pH 9.1 was then pipetted onto the sections in the through. Thus the sections were placed between the two solutions, in a region of continuous cobalt precipitation. After various periods the filter with the sections was rinsed and processed in the usual manner. The precipitate was diffusely distributed in the sections but for a marked tendency to stain nuclei and/or nucleoli. This result was obtained with: human skin, toad kidney and mouse stomach, kidney and duodenum. The human skin was fixed in glutaraldehyde and the toad kidney was fixed by glutaraldehyde perfusion, the remaining tissues were fresh frozen.

d) The possibility of unspecific staining due to an exchange of cobalt against zinc in *e.g.* the islets of pancreas (FAND, LEVINE and ERWIN, 1959; BLEYL, 1964) has been suggested. However, with the present method the islet cells of the rat pancreas did not stain whereas the exocrine part stained intensely. Contrary to the findings of BLEYL (1964) staining capillaries were found in the islets.

Discussion

A well known principle in enzyme histochemistry is the precipitation of one component present in the solution with a reaction product formed by the activity of the enzyme in the submerged tissue section. The sites of precipitation are interpreted as being close to the enzyme. This approach, when applied to the reaction catalyzed by CA ($CO_2 + H_2O \rightleftharpoons H_2CO_3$) presents special difficulties due to the rapidity of the uncatalyzed reaction. The straightforward thing to do would seem to be to arrange for the precipitation of a suitable carbonate. The pH range where enough carbonate ions are formed and precipitation takes place is determined by the pKa of HCO_3^-, 10.3. However, above pH 10 the uncatalyzed reaction is predominantly: $CO_2 + OH^- \rightleftharpoons HCO_3^-$ and very rapid (see KERN, 1960 for references). Thus unspecific reaction occurs already after a short time of incubation. If one chooses a lower pH, another factor becomes limiting. At the sites of enzyme activity the concentration of carbonate formed must exceed the solubility product. At low pH but little CO_3^{--} forms, high concentrations of the precipitable cation are needed and absorption of cation to the tissue components causes unspecific staining. The author has made a great many experiments with a method according to the conventional principles (precipitating Pb, Co, Ba or Sr) around pH 10 and at 1° C (to slow down the uncatalyzed reaction). However, the bad reproducibility of the staining reactions obtained made the method unsuitable. This was mainly caused by the time lag between establishment of supersaturation and the precipitation of carbonate from the supersaturated solution. During this time lag even the uncatalyzed reaction runs almost to completion.

In the method of HÄUSLER (1958), WALDEYER and HÄUSLER (1959) and in the present modification the phenomena close to the fluid surface are the important ones. They can be written as follows:

$$\begin{array}{ccccccccc} & & & & & & & \overset{CA}{\uparrow} & \\ CO_3^- + H^+ & \rightleftharpoons & HCO_3^- + H^+ & \rightleftharpoons & H_2CO_3 & \rightleftharpoons & CO_2 + H_2O & & \\ + & & + & & & & & & \\ OH^- & & OH^- & & & & & & \\ \uparrow\downarrow & & \uparrow\downarrow & & & & & & \\ H_2O & & H_2O & & & & & & \end{array}$$

As shown in control experiment b) the loss of CO_2 is the essential reaction which forces the reaction to the right resulting in a consumption of HCO_3^-, CO_3^{--} and a production of OH^-. If a section is floated on the surface of the medium there will be a higher rate of production of OH^- ions at sites of CA activity, because the loss of CO_2 is catalyzed.

When incubation mixture is prepared by pouring the bicarbonate solution over the acid $CoSO_4$ the equilibrium between carbon dioxide and carbonate in the solution will be nearly completed in a few seconds (KERN, 1960). If then carbon dioxide is allowed to leave the solution, different concentrations of the reactants will be found in the surface layer and beneath. In this way the relatively rapid carbonate — carbon dioxide reaction is followed by another reaction: the establishment *and maintenance* by enzymatic activity of local OH^- accumulations which are utilized in the histochemical methods.

Assume that enough cobalt ions are present in the solutions. The concentration of OH^- ions will eventually exceed the solubility product of cobalt hydroxide (10^{-16}). With a cobalt concentration around 10^{-3} this occurs around pH 7.5. It is probable that the reaction product in the method of HÄUSLER (1958) is cobalt hydroxide or a more or less complex basic carbonate. Possibly also sulfate takes a part.

HÄUSLER (1958) himself gives an explanation for his reaction which in fact assumes that removal of CO_2 from the solution by release into the air causes an increase in the concentration of CO_3^{--} ions which precipitate as $CoCO_3$. This factor might be of some importance but at the actual pH the local accumulation of OH^- is probably more significant.

The present method is also explained by the same OH^- producing reaction mechanism. In addition, some protons used in the formation of H_2CO_3 may derive from $H_2PO_4^-$. Thus, either OH^- or HPO_4^{--} are continuously produced and more or less stable local excess concentrations of these products will be established at the sites of enzyme action.

Now the experiments show that inclusion of KH_2PO_4 in the medium (up to 10 ml 1/15 *M* KH_2PO_4/57 ml) greatly shortens the incubation time necessary for a cobalt compound to precipitate. This is not only due to a shift in the pH range over which the reaction proceeds. While with low phosphate concentrations the precipitate probably approaches the same cobalt complex as in the method of HÄUSLER except for the possible inclusion of less SO_4^{--}, the ratio of P/Co of the precipitate increases with the phosphate concentration. This precipitate must have a lower solubility product than the complex formed in the absence of phosphate. It is possible that this fact is sufficient to explain most of the phosphate effect. The phosphate ions present do not activate the enzyme (KERNOHAN, 1965). Another factor could be the much slower diffusion of HPO_4^{--} from the site of production than of OH^-, which would favour the establishment of local excess concentrations at the enzyme sites. This list of possibilities is not exhaustive.

The fate of ions produced at the sites of enzyme activity will depend on the presence and concentration of precipitable material. If no cobalt is present, they will diffuse away. Some will do this also in the presence of cobalt but some will be precipitated and consumed this way. If conditions for precipitation are very favourable, the *precipitation threshold* is rapidly exceeded and one would expect

a sharper localization. It is possible that the good localization observed with phosphate is also due to the low diffusibility of HPO_4^{--}.

Once precipitation has started, the rate of the catalyzed reaction may or may not be limiting for the rate of further deposition of material and for the time used for the *visualization threshold* to be reached. With very high local enzyme activities, other factors can become rate-limiting. By keeping all reactants at the highest possible concentration, one maximises two things: the sensitivity and the range of enzyme activity over which a relation between rate of deposition and activity exists.

The highest suitable concentration of cobalt is limited by visible unspecific binding to the tissue. At the cobalt concentration used a phosphate concentration could be found (about 10^{-2} *M* with sections from the skin) further elevation of which did not shorten the appearance time of the staining reaction. This does not necessarily mean that in these sections there was 100 per cent efficiency in the utilization of the enzymatic reaction since for instance inhibiting impurities in the phosphate buffer (KERNOHAN, FORREST and ROUGHTON, 1963) would give the same result.

Inhibition of enzyme activity increases the time necessary for visible amounts of cobalt to accumulate. The least concentration of inhibitor which inhibits a visible staining reaction depends on the degree of local catalytic activity, the time of incubation and the sensitivity of the medium set by the concentration of phosphate. The concentration of acetazolamide necessary to inhibit the staining reaction in the methods of HÄUSLER (1958) and WALDEYER and HÄUSLER (1959) is generally stated to be 5×10^{-3} *M*. As MUTHER (1966) has pointed out these high concentrations of acetazolamide might withdraw cobalt by formation of an inhibitor-cobalt complex. This would abolish the staining reaction and simulate enzyme inhibition. In the present method, however, this mechanism can not possibly act because the inhibitor concentration is more than one thousand times less than of cobalt. Therefore if a staining site is found which is inhibited only by concentrations of the same order as that of cobalt it would seem logical to reduce phosphate concentration, temperature or incubation time to see whether the sensitivity to inhibition can be increased so far that concentrations of inhibitor far below that of cobalt are sufficient. The author has never found any site which was resistant to such concentration of acetazolamide if conditions were chosen right.

Before the sites of specific staining reaction can be identified with sites of CA activity the possibilities of errors must be considered.

Using immunological methods there is an easily measured loss of CA activity by diffusion from fresh frozen sections of human tissues floated on the surface of a solution (HANSSON, to be published). Whether such a loss also occurs in the method of KORHONEN and KORHONEN (1965b) where fixed sections are soaked before staining is not known. With the present method fixation as recommended by KORHONEN and KORHONEN (1965b) did not shorten the time for visible staining which indicates that it did not preserve any extra, active enzyme in the sections.

Thus unfixed freeze-dried sections are to be preferred for the demonstration of weak CA activity. Since it is not known if all sites of the enzyme lose equally

much by leaching during the incubation, selective loss of enzyme activity must be reckoned with NACHLAS, PRINN and SELIGMAN (1956).

The method of HÄUSLER may give a nuclear staining which has been discussed by KORHONEN, NÄÄTÄNEN and HYYPPÄ (1964). They regarded it as an artefact. Also in the present method there may be a nuclear or nucleolar staining in partly inhibited sections, absent in fully inhibited sections. CA has been found in the nuclear fraction of some cells (KARLER and WOODBURRY, 1959) but the histochemical finding may also be due to adsorbtion of the diffusible enzyme (see NACHLAS, YOUNG and SELIGMAN, 1957, for references). However, as shown in control experiment c) nuclear and nucleolar staining can also occur in the absence of enzymatic activity, if pH and the concentration of cobalt ions are high enough. Now, why does nuclear staining appear only sometimes? Assume that the CA activity is low due to a small local concentration of enzyme or a partial inhibition by inhibitors or fixation. Under these conditions the precipitation threshold may never be reached at the site of enzyme and the reaction product (OH^- and/or HPO_4^{--}) will diffuse away from the site of production and raise the pH in *e.g.* adjacent nuclei. False localization due to binding of cobalt may then appear. Structures which stain in control experiments of type c) without any substrate, must be regarded as non-enzymatic sites until the opposite has been proven. This error and the diffuse spreading of the reaction product should decrease, the more rapidly the precipitation threshold is reached, provided that enough cobalt reaches the enzyme sites.

The spatial resolution of the staining reaction is hard to evaluate from histological evidence alone, but it can be quite high. There is an easily distinguishable pattern of staining in single cells, see *e.g.* some panels of Fig. 6, but whether the sites of staining here are identical with the enzyme sites can only be decided by extravenous evidence, for instance fractionation of subcellular components.

In certain tissues one has a rather good understanding of the physiological significance of the enzyme, *e.g.* in the red cells and in many glands. With the sensitive method presented here it is now possible to find enzyme containing cells which comprise only a minor part of the tissue mass and which might have escaped attention during investigations of the concentration of the enzyme in tissue homogenates. It will now be necessary to construct experiments to estimate the role played by the enzyme at these minor sites and eventually evaluate the therapeutic potentials of carbonic anhydrase inhibitors with regard to their attack on these cell systems.

Acknowledgements. I am grateful to Professor ERNST BÁRÁNY for his invaluable criticism and help; to Mrs. ANNELIE FORSBERG and to Mrs. ASTA PALIS for technical assistance; to Lederle Laboratories for providing the Diamox® and CL 13850; and to Merck, Sharp & Dohme for providing Chlotride®.

References

BLEYL, U.: Zur Spezifität des histochemischen Carboanhydratasenachweises im Inselorgan der Bauchspeicheldrüse. Histochemie **4**, 286—311 (1964).

DATTA, P. K., and T. H. SHEPARD: Intracellular localization of carbonic anhydrase in rat liver and kidney tissues. Arch. Biochem. **81**, 124—129 (1959).

DAVENPORT, H. W.: Gastric carbonic anhydrase. J. Physiol. (Lond.) **97**, 32—43 (1939).

FAND, S. B., H. J. LEVINE, and H. L. ERWIN: A reappraisal of the histochemical method for carbonic anhydrase. J. Histochem. Cytochem. **7**, 27—33 (1959).

GIACOBINI, E.: A cytochemical study of the localization of carbonic anhydrase in the nervous system. J. Neurochem. **9**, 169—177 (1962).

HANSSON, H. P. J.: Demonstration of carbonic anhydrase by means of fluorescent antibodies in human erythrocytes. Life Sci. **4**, 965—968 (1965).

HÄUSLER, G.: Zur Technik und Spezifität des histochemischen Carboanhydrasenachweises im Modellversuch und in Gewebsschnitten von Rattennieren. Histochemie **1**, 29—47 (1958).

KARLER, R., and D. M. WOODBURY: Intracellular distribution of carbonic anhydrase. Biochem. J. **75**, 538—543 (1959).

KERN, D. M.: The hydration of carbon dioxide. J. Chem. Educat. **37**, 14—23 (1960).

KERNOHAN, J. C.: The pH-activity curve of bovine carbonic anhydrase and its relationship to the inhibition of the enzyme by anions. Biochim. biophys. Acta (Amst.) **96**, 304—317 (1965).

— W. W. FORREST, and F. J. W. ROUGHTON: The activity of concentrated solutions of carbonic anhydrase. Biochim. biophys. Acta (Amst.) **67**, 31—41 (1963).

KORHONEN, E., and L. K. KORHONEN: Histochemical demonstration of carbonic anhydrase activity in the eyes of rat and mouse. Acta ophthal. (Kbh.) **43**, 475—481 (1965).

— — Histochemical demonstration of carbonic anhydrase activity in mast cells. Experientia (Basel) **21**, 628 (1965a).

— — Electrophoretic and histochemical studies of carbonic anhydrase activity. Histochemie **5**, 279—288 (1965b).

KORHONEN, L. K., E. NÄÄTÄNEN, and M. HYYPPÄ: A histochemical study of carbonic anhydrase in some parts of the mouse brain. Acta histochem. (Jena) **18**, 336—347 (1964).

MAREN, T. H.: Carbonic anhydrase inhibition. V. N^5-substituted 2-acetylamino-1,3,4-thiadiazole-5-sulfonamides: Metabolic conversion and use as control substances. J. Pharmacol. exp. Ther. **117**, 385—401 (1956).

— V. I. ASH, and E. M. BAILY jr.: Carbonic anhydrase inhibition. II. A method for determination of carbonic anhydrase inhibitors, particularly of Diamox®. Bull. Hopkins Hosp. **95**, 244—255 (1954).

MUSTAKALLIO, K. K., J. RAEKALLIO, and E. RAEKALLIO: The histochemical demonstration of carbonic anhydrase. An attempt to localize its inhibition by acetazolamide (Diamox®) in rat kidney. Ann. Med. exp. Fenn. **38**, 247—251 (1960).

MUTHER, T. F.: On the non-specificity of histochemical methods for carbonic anhydrase. Fed. Proc. **25**, 320 (1966).

NACHLAS, M. M., W. PRINN, and A. M. SELIGMAN: Quantitative estimation of lyo- and desmoenzymes in tissue sections with and without fixation. J. biophys. biochem. Cytol. **2**, 487—502 (1956).

— A. C. YOUNG, and A. M. SELIGMAN: Problems of enzymatic localization by chemical reactions applied to tissue sections. J. Histochem. Cytochem. **5**, 565—583 (1957).

WALDEYER, A., and G. HÄUSLER: Histochemische Studien über die Carboanhydraseaktivität der Samenwege und ihrer Anhangdrüsen von Mus rattus. Acta biol. med. germ. **2**, 568—589 (1959).

WISTRAND, P. J.: Comparison by direct measurement of sulfonamide carbonic anhydrase inhibition in whole cells and in enzyme solutions. In: Drugs and enzymes. Proc. of the 2nd Internat. Pharmacological Meeting, Prague 1963, vol. 2, p. 176—186. Oxford: Pergamon Press 1965.

—, and S. N. RAO: Immunological and kinetic properties of carbonic anhydrases from various tissues. Biochem. biophys. Acta (Amst.) (in press) (1967).

Dr. H. P. J. HANSSON
Dept. of Pharmacology, Univ. of Uppsala
Dag Hammarskjölds väg 19
Uppsala/Sweden