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CARBONIC ANHYDRASE FROM *NEISSERIA SICCA*, STRAIN 6021

I. BACTERIAL GROWTH AND PURIFICATION OF THE ENZYME

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SUMMARY

1. Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) from *Neisseria sicca*, strain 6021, has been studied. The bacteria have been grown in liquid cultures and the growth conditions have been varied in order to improve enzyme production.

2. The enzyme can be released from the bacteria by shaking the cells with glycine–NaOH buffer, pH 9–10. The extract has been fractionated and a procedure consisting of three ion-exchange chromatography steps is described giving more than 100-fold purification.

3. The purified enzyme appears to be homogeneous on ultracentrifugation and in polyacrylamide gel electrophoresis. No evidence has been obtained for the existence of more than one form of the bacterial enzyme; a situation in contrast to that for mammalian carbonic anhydrases where polymorphic patterns are often encountered.

INTRODUCTION

During the last few years considerable efforts have been spent on investigations of the properties and function of the enzyme carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1). Significant progress, recently reviewed[1,2], has been achieved about the knowledge of the kinetic behavior of the enzyme as well as of structural features. Studies have mostly been concerned with carbonic anhydrases from mammalian erythrocytes, which for a considerable period of time were the only forms of the enzyme sufficiently purified and characterized. More recently a few non-mammalian animal carbonic anhydrases have also been isolated in pure form[3,4] as well as the plant enzymes from parsley[5], pea[6] and spinach leaves[7].

The present and companion paper are concerned with a carbonic anhydrase of bacterial origin and represent an attempt to introduce an enzyme molecule which could be varied by genetic alterations. The presence of carbonic anhydrase activity in bacteria was first reported by Veitch and Blankenship[8] who found enzymic activity in several strains belonging to the genus *Neisseria*. The highest activity was found in

Abbreviation: SE-Sephadex, sulfoethyl Sephadex.

Neisseria sicca, strain 6021, and this strain has been used in this study. The present paper describes the conditions used for bacterial growth, extraction of the enzyme from bacterial cells and its purification from this extract. The purified enzyme shows a high degree of homogeneity on ultracentrifugation and polyacrylamide gel electrophoresis. The properties of the purified enzyme are described in the following paper[9].

MATERIALS AND METHODS

Bacterium

Neisseria sicca, strain 6021, was obtained from Professor M. J. Pelczar, Department of Microbiology, University of Maryland, College Park, Md., U.S.A. The strain was preserved in a lyophilized state after growth in a heart infusion broth medium (see the section *Growth conditions*).

Enzyme activity measurements

The rate of the carbon dioxide hydration reaction was measured with a Durrum–Gibson stopped-flow spectrophotometer equipped with a 2-cm cuvette. An aliquot of enzyme solution (usually 100 μl) was mixed with 5.0 ml of 0.04 M Veronal–H_2SO_4 buffer, pH 7.9 or 8.3 (20 °C), also containing $1.13 \cdot 10^{-5}$ M Phenol red. This solution was injected into the cuvette together with an equal amount of water saturated with CO_2 at 25 °C. The reaction was followed at 20 °C and 560 nm. Enzyme activity was estimated as $[(t_T)^{-1} - (t_S)^{-1}] \times F$, where t_T and t_S are the times in seconds required for a change in transmission from 50 to 70% in the presence and absence of enzyme, respectively, and F is a dilution factor (concentration of enzyme in original solution to that in the reaction mixture). The figure obtained in this way divided by $A_{280\ nm}^{1\ cm}$ for the original protein solution is defined as specific enzyme activity.

Extraction of carbonic anhydrase from bacterial cells

It was found that carbonic anhydrase could be released into the solution by shaking the cells with buffer. Release of enzymic activity was observed to take place in the following buffers: 0.1 M phosphate, pH 7.0 and 8.5; 0.1 M Tris–HCl, pH 8 and 9; glycine–NaOH, pH 9–10, $I = 0.1$. In general, an increased release of enzymic activity with increase in pH could be noted but the result was also influenced by the

TABLE I

INFLUENCE OF pH AND SODIUM SALTS ON THE RELEASE OF CARBONIC ANHYDRASE FROM *Neisseria sicca*

Bacterial cells from 75 ml of culture medium were suspended in 1 ml of glycine–NaOH buffer, $I = 0.10$, containing different amounts of NaCl or Na_2SO_4. The cells were shaken for 1 h at 37 °C on a rotatory shakeboard at 60 rev./min.

Total ionic strength (M)	*Electrolyte added*	*Enzyme activity at*		
		pH 10.0	*pH 9.6*	*pH 9.0*
0.10	—	104	45	26
0.20	NaCl	118	63	53
0.30	NaCl	91	75	52
0.25	Na_2SO_4	80	70	49
0.40	Na_2SO_4	84	77	53

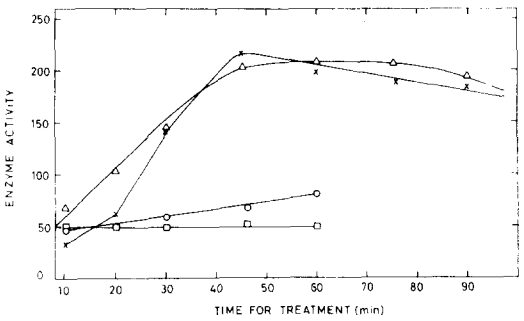


Fig. 1. Release of carbonic anhydrase from *Neisseria sicca* as a function of time and temperature Bacterial cells were treated at various temperatures by shaking in glycine–NaOH buffer, pH 10 $I = 0.1$, for varying periods of time. The suspensions were then centrifuged and the supernatant removed and assayed for carbonic anhydrase activity. ×—×, 45 °C; △—△, 37 °C; ○—○ 22 °C; □—□, 8–10 °C.

ionic composition of the solution. The best results were obtained with glycine–NaOH buffer and Table I illustrates the effects of pH and sodium salts for this particular buffer. Fig. 1 shows the release of enzymic activity in glycine–NaOH, pH 10, $I = 0.1$, as a function of time at various temperatures. From the results in Table I and Fig. 1 the following conditions were chosen as a standard procedure: Bacterial cells from a 20-l culture are washed two times with distilled water and cold glycine–NaOH buffer pH 10, $I = 0.1$. The cells are suspended in 600 ml of the same buffer and shaken in a 2-l separating funnel on a rotatory shaker (60 rev./min) at 37 °C for 1 h. The suspension is centrifuged in a Sorval RC-2 centrifuge at 14 000 × *g* for 45 min and the supernatant is removed and used for the purification of carbonic anhydrase.

Lysozyme (EC 3.2.1.17) which is known to be effective for the release of enzymes located in the outer layers of Gram-negative bacteria was found to be without effect on the extraction procedure described above. The release of carbonic anhydrase from *Neisseria* cells can also be achieved by treatment with ultrasonics (*cf.* ref. 10). The extract obtained in this way appears, however, to be more complex than the glycine–NaOH extract and for that reason less suitable as the starting material for enzyme purification. Sonification of the precipitate from glycine–NaOH extraction does not result in any further release of enzyme activity which would suggest that the treatment with glycine–NaOH buffer is an efficient procedure for the extraction of the enzyme from the bacterial cells.

Growth conditions

The bacteria were grown in liquid cultures at 37 °C. Studies of the influence of various factors on growth and enzyme synthesis were performed with cultures containing 1 l of medium while the production of bacteria for fractionation and purification of carbonic anhydrase was carried out in culture flasks with 20 l of medium. Aliquots of the bacterial suspension were withdrawn at various times. These were used for the estimation of growth by absorbance measurements at 600 nm and for the assay of carbonic anhydrase activity after release of the enzyme from the bacterial cells as described in the previous section.

Most experiments were carried out at a constant pH of 7.1 which was maintained with a titrator (type TTT1c, Radiometer, Copenhagen) equipped with an

automatic valve regulating the addition of NaOH to the culture flask. The pH electrode used was a combination electrode of the size 10 mm × 400 mm (Model 2-Ea 120 UX, Metrohm AG. Herisau, Switzerland) which prior to its insertion in the culture flask was sterilized by the following procedure: After washing with a commercial sterilizing soap (sodium *o*-phenyl phenolate) it was rinsed with sterile water, soaked in a 0.5% solution of iodine (Jodopax, Ferrosan, Malmö, Sweden) and washed with 70% ethanol. To remove traces of iodine it was kept in sterile water and finally rapidly burnt off and inserted in the autoclaved culture flask.

The culture flasks were also equipped with a stirring device and a gas inlet permitting air to bubble through the bacterial suspension. The gas flow was sterilized through a wash bottle filled with cotton which had been autoclaved and then dried by lowering the pressure of the autoclave.

A mixture of 25 g heart infusion broth (Difco) + 3 g yeast extract (Difco) + 2 g glucose per l was used as a basic medium (*cf.* ref. 11). Sterilization was carried out by autoclaving. The glucose was autoclaved separately in a small amount of distilled water and after cooling to about 37 °C added to the culture flask. The growth and enzyme synthesis was studied after various supplementary additions to this basic medium and these chemicals were also autoclaved separately as specified in Results and added to the culture flasks afterwards, together with the glucose.

Harvesting of cells from the 20-l cultures was carried out by centrifugation in an International Serum Centrifuge, Model 13 l, at 2000 × *g* and 15–20 °C for 1 h or at 14 000 × *g* and 2–5 °C in a Sorvall RC-2 centrifuge equipped with the attachment for continuous flow.

Fractionation of carbonic anhydrase

Carbonic anhydrase was extracted from the harvested cells with glycine–NaOH buffer, pH 10, $I = 0.1$, as described in a previous section. This extract was dialyzed and used as the starting material in fractionation experiments.

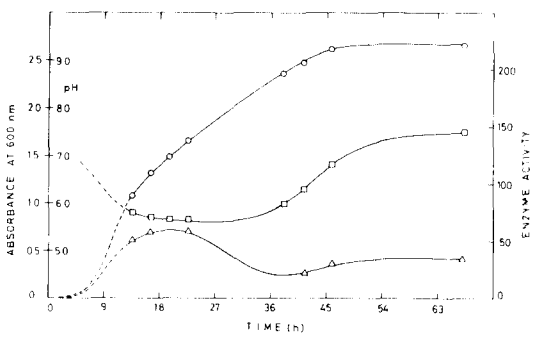
Ion-exchange chromatography was carried out on DEAE-, CM-, and sulfoethyl (SE-) Sephadexes (Pharmacia, Uppsala, Sweden). Detailed experimental conditions are described in the captions to Figs 4–6. Gel filtrations were performed on Sephadex G-75 (Pharmacia, Uppsala) in 0.1 M Tris–H_2SO_4, pH 7.5. Zone electrophoresis in phosphate buffer, pH 6.5, $I = 0.05$, and phosphate–borate buffer, pH 8.2, $I = 0.05$, was carried out in a jacketed and cooled (≤ 6 °C) vertical column (10 mm × 750 mm) packed with ethanolyzed cellulose powder (Grycksbo Pappersbruk, Grycksbo, Sweden)[12].

The eluate from the columns was collected with a time-regulated fraction collector. Absorbances at 260 and 280 nm were measured on individual fractions with a Zeiss PMQ II spectrophotometer and carbonic anhydrase activity assayed as described under the section *Enzyme activity measurements*.

RESULTS

Growth conditions

The effects on enzyme synthesis and bacterial growth by varying the composition of the medium are summarized in Figs 2–3 and Table II. It has already been reported[13] that the metabolism of glucose by *Neisseria sicca* is accompanied by proton



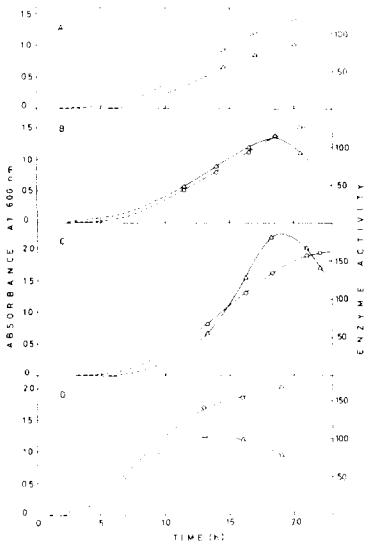


Fig. 2. Bacterial growth, synthesis of carbonic anhydrase, and change in pH in a culture of *Neisseria sicca* in basic medium + 5 mM phosphate, pH 7.1. Aliquots of 50 ml were withdrawn from the bacterial culture at various times and used for pH determination, estimation of cell density by absorbance measurement at 600 nm, and determination of carbonic anhydrase activity after release of the enzyme from bacterial cells by glycine–NaOH buffer, pH 10, $I = 0.1$. Composition of the basic medium and experimental details are described in Materials and Methods. ○—○, absorbance at 600 nm (1-cm cell); △—△, enzyme activity; □—□, pH.

Fig. 3. Bacterial growth and synthesis of carbonic anhydrase in cultures of *Neisseria sicca* at pH 7.1 in growth media of various compositions. The pH was maintained constant by the automatic addition of NaOH. ○—○, cell density estimated by absorbance measurement at 600 nm (1-cm cell); △—△, enzyme activity. For details see the caption to Fig. 2. The metal ion mixture used is specified in Table II. A. Basic medium + 5 mM phosphate, pH 7.1. B. Basic medium + 5 mM phosphate, pH 7.1, + 2 μM $ZnSO_4$. C. Basic medium + 5 mM phosphate, pH 7.1, + 2 μM $ZnSO_4$ + metal ion mixture. D. Basic medium + 5 mM phosphate, pH 7.1, + 2 μM $ZnSO_4$ + metal ion mixture + 7.5 mM NH_4Cl.

TABLE II

INFLUENCE OF GROWTH CONDITIONS ON THE AMOUNT OF CARBONIC ANHYDRASE IN CULTURES OF *Neisseria sicca*

The bacterium was grown in the basic medium (25 g heart infusion broth + 3 g yeast extract + 2 g glucose per l) + 5 mM phosphate buffer, pH 7.1, with supplementary additions as specified below. In Expts 2–7 the pH during growth was maintained at 7.1 by the automatic addition of NaOH. Composition of metal ion mixture: 2 mM $MgSO_4$ + 1 μM $MnCl_2$ + 0.8 mM $CaCl_2$. Concentrations given are final values.

Expt No.	*pH control*	*Supplementary additions*			*Maximum enzyme activity*
		$ZnSO_4$ (μM)	*Metal ion mixture*	*NH_4Cl (mM)*	
1	—	—	—	—	60
2	+	—	—	—	85
3	+	1	—	—	103
4	+	2	—	—	113
5	+	4	—	—	78
6	+	2	+	—	185
7	+	2	+	7.5	103

production. This was also found to be valid for the strain used in the present investigation. Growth in the basic medium without any control of the pH leads to a marked decrease in pH from the initial value of 7.1. Attempts to counteract this effect were made by adding phosphate buffer to the basic medium (see Fig. 2) but even with the highest concentration tested (50 mM) the decrease still amounted to more than 1 pH unit and no significant increase in maximum enzyme activity was obtained. Keeping the pH constant by automatic addition of NaOH apparently results in an increased yield of enzyme (see Fig. 3A and Table II).

Carbonic anhydrases from other sources have been shown to contain a firmly bound zinc ion which is essential for enzymic activity[14]. As is evident from Fig. 3B and Table II, the addition of small amounts of $ZnSO_4$ to the growth medium increases the yield of carbonic anhydrase activity. Catlin and Schloer[15] have described a medium for *N. meningitidis* containing $MgSO_4$, $MnCl_2$, and $CaCl_2$ and the introduction of these metal salts in the medium used here obviously promotes the yield of enzyme (Fig. 3C). The presence of the inorganic nitrogen source, NH_4Cl, desirable in the case of meningococci[15], was found to lower the yield of carbonic anhydrase (see Fig. 3D and Table II).

An increase of the glucose concentration in the medium from 2 to 4 g/l was found to increase the growth rate slightly.

From these results the following medium was chosen for the routine production of bacteria for the purpose of purifying the carbonic anhydrase. The figures given below represent final concentrations. The components A to E were dissolved in distilled water, autoclaved separately and mixed together after cooling to 37 °C.

A	Heart Infusion Broth (Difco)	25 g/l
	Yeast Extract (Difco)	3 g/l
	Na_2HPO_4	3.6 mM
	KH_2PO_4	1.4 mM
	Antifoam A (Midlands Silicons)	Trace amount
B	Glucose	4 g/l
C	$ZnSO_4$	2 μM
D	$MgSO_4$	2 mM
	$MnCl_2$	1 μM
E	$CaCl_2$	0.8 mM

A typical growth curve under these conditions is illustrated in Fig. 3C. The relation between enzyme activity and amount of bacterial cells (expressed as absorbance at 600 nm) varies considerably along the growth curve. Enzymic activity increases rapidly to a maximum value and then starts to decrease while the density of the bacterial suspension is still increasing. Harvesting of cells was carried out at about the point of maximum enzyme activity which occurs 15–20 h after initiation of growth and was found to correspond to a density of the bacterial population of approx. 1.8 absorbance units at 600 nm (1-cm cell). Maintenance of the pH at 7.1 during growth of a 20-l culture required the addition of 400–500 ml of 2 M NaOH.

Preliminary experiments showed that the bacteria grow faster when, instead of air, an air/CO_2 mixture (10% CO_2) is bubbled through the suspension. However, only a very low amount of enzymic activity could be found in the extract from cells grown under these conditions.

Purification of carbonic anhydrase

From fractionation experiments with the bacterial carbonic anhydrase the fol-

TABLE III

PURIFICATION OF CARBONIC ANHYDRASE FROM *Neisseria sicca*

The data refer to a 20-l culture of bacterial cells. The purification steps in the first column refer to the procedure described in section Results. Total protein is expressed as the product of volume (ml) and absorbance at 280 nm. Specific enzyme activity is defined as under Materials and Methods. Total enzyme activity is the product of total protein and specific enzyme activity. It has been expressed as a percent, assuming 100% yield from *Step 1*.

Enzyme solution from step No.	*Vol. (ml)*	$A_{280\ nm}$ *(1-cm cell)*	$A_{260\ nm}$ *(1-cm cell)*	*Total protein*	*Specific enzyme activity*	*Total enzyme activity (%)*
1	400	11.13	18.10	4450	2.70	100
2	740	0.222	0.221	164	58.1	79
3	44	0.528	0.427	23.2	514	99
4	9.5	0.655	0.420	6.22	1560	81

lowing sequence of steps is suggested as a purification procedure. The yield of enzyme and the degree of purification achieved in each step are illustrated in Table III.

Step 1. Cells from a 20-l culture are harvested and extracted with glycine–NaOH buffer as described in Materials and Methods.

Step 2. The enzyme solution is dialyzed against 0.2 M Tris–H_2SO_4 buffer, pH 7.5, at 8–10 °C for two days and then filtered through a column packed with DEAE-Sephadex A-50 and pre-equilibrated with the same buffer. Details of experimental conditions are given in the caption to Fig. 4. The carbonic anhydrase comes through only

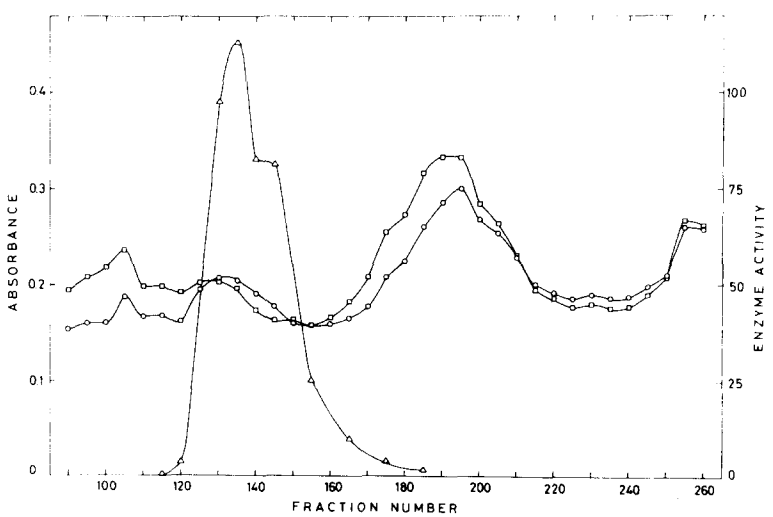


Fig. 4. Fractionation of a crude extract of bacterial carbonic anhydrase on DEAE-Sephadex A-50 (corresponding to *Step 2* in the purification procedure described in Results). Column dimensions: 80 mm × 400 mm. Temperature: 8 –10 °C. Fraction volume: about 16 ml. Flow rate: 150 ml/h. ○—○, absorbance at 280 nm (1-cm cell); □—□, absorbance at 260 nm (1-cm cell); △—△, enzyme activity. 400 ml of enzyme extract from *Step 1* in the purification procedure were dialyzed against 0.2 M Tris–H_2SO_4, pH 7.5, and filtered through the column which had previously been equilibrated with the same buffer. The enzyme passes almost unretarded through the column while material with absorption at 260 nm is retarded. Fractions Nos 120 to 165 were pooled and rechromatographed as described in Fig. 5.

slightly retarded. A yellow pigment is attached to the top of the column and large amounts of material with a strong absorbance at 260 nm (presumably nucleic acids) are retarded on the column. The fractions containing carbonic anhydrase activity are pooled and rechromatographed as described under *Step 3*.

A few other methods were unsuccessfully tried for further purification of the enzyme derived from *Step 2*. Zone electrophoresis on cellulose columns, a technique routinely used in this laboratory for the preparation of carbonic anhydrases from human and bovine erythrocytes, turned out to be of little value in the case of the bacterial enzyme. Sometimes the recovery of enzyme activity was very low (20–25%) and a significant degree of purification was never achieved.

Step 3. The enzyme solution from the DEAE-chromatography is further fractionated by ion-exchange chromatography on CM-Sephadex C-50. The enzyme is attached to the ion-exchange column in 0.2 M Tris–H_2SO_4 buffer, pH 7.5, and subsequently eluted with a concentration gradient as described in the caption to Fig. 5. The enzyme activity coincided roughly, but not exactly, with a peak of ultraviolet absorption as can be seen by careful inspection of the diagram. This tendency of activity distribution towards the trailing section of an ultraviolet peak is the usual outcome of this chromatographic step.

Step 4. The enzyme solution from the preceding step is dialyzed against 0.15 M Tris–H_2SO_4, pH 7.5, and filtered through a column of SE-Sephadex C-50. Some material with predominant ultraviolet absorption at 260 nm goes through the column as shown in Fig. 6 while the enzyme remains attached and is subsequently eluted as an apparently homogeneous peak with a concentration gradient of 1.0 M Tris–H_2SO_4, pH 7.5. This chromatography has been carried out with a similar result using 0.075 M sodium phosphate, pH 7.5, as the starting buffer and a gradient from this buffer to 0.3 M sodium phosphate of the same pH. The enzyme derived by the SE-Sephadex

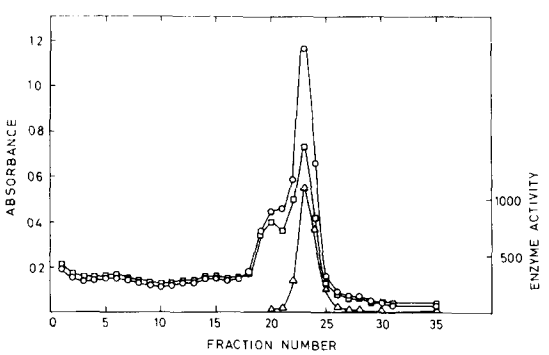


Fig. 5. Chromatography of partially purified bacterial carbonic anhydrase on CM-Sephadex C-50 (corresponding to *Step 3* in the purification procedure described in Results). Column dimensions: 15 mm × 150 mm. Temperature: 8–10 °C. Fraction volume: about 6 ml. Flow rate: 36 ml/h. ○—○, absorbance at 280 nm (1-cm cell); □—□, absorbance at 260 nm (1-cm cell); △—△, enzyme activity. 740 ml of enzyme solution in 0.2 M Tris–H_2SO_4, pH 7.5, from *Step 2* (Fig. 4) were applied to the column which had previously been equilibrated with the same buffer. Considerable amounts of ultraviolet absorbing material ran through the column (not shown in the diagram) while the enzyme remained attached. After washing of the column with 25 ml of 0.2 M Tris–H_2SO_4, pH 7.5, the enzyme was eluted with a concave concentration gradient from 0.2 M to 1.0 M Tris–H_2SO_4, pH 7.5. Fraction Nos 21 to 27 were pooled and used for rechromatography on SE-Sephadex (see Fig. 6).

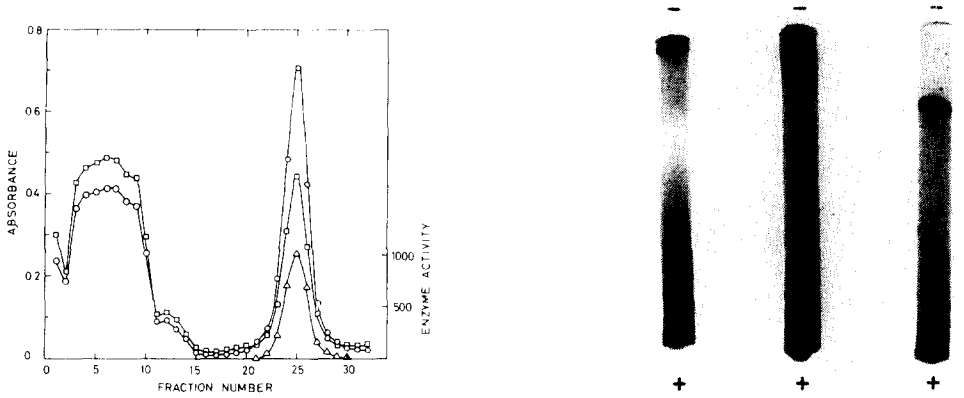


Fig. 6. Chromatography of partially purified bacterial carbonic anhydrase on SE-Sephadex C-50 (corresponding to *Step 4* in the purification procedure described in Results). Column dimensions: 16 mm × 200 mm. Temperature: 8–10 °C. Fraction volume: 3–4 ml. Flow rate: 15 ml/h. ○—○, absorbance at 280 nm (1-cm cell); □—□, absorbance at 260 nm (1-cm cell); △—△, enzyme activity. 44 ml of enzyme solution from *Step 3* (Fig. 5) were dialyzed against 0.15 M Tris–H_2SO_4, pH 7.5, and applied to the column which had previously been equilibrated with the same buffer. After washing of the column with 10 ml of buffer the enzyme was eluted with a concave concentration gradient from 0.15 M to 1.0 M Tris–H_2SO_4, pH 7.5.

Fig. 7. Disc electrophoresis pattern of purified bacterial carbonic anhydrase. The electrophoresis was carried out on 3.5% polyacrylamide gel in Tris–glycine buffer, pH 9.5 (ref. 17, p. 372) for 10 to 35 min at 5 mA. Staining was performed with 0.1% Naphthalene black in 10% trichloroacetic acid.

chromatography was often checked for homogeneity by gel filtration on Sephadex G-75 in 0.1 M Tris–H_2SO_4, pH 7.5.

Homogeneity of the purified enzyme

The enzyme solution derived from the final step in the purification procedure above shows a ratio in absorbances at 280 nm and 260 nm of 1.65, suggesting that the enzyme has become efficiently freed of contaminating nucleic acids[16]. Polyacrylamide gel electrophoresis[17] gives a single protein band (Fig. 7) and a homogeneous peak was observed during sedimentation in the ultracentrifuge (Fig. 8). Gel filtration on Sephadex G-75 in 0.1 M Tris–H_2SO_4, pH 7.5, gives a single protein peak containing the enzyme activity.

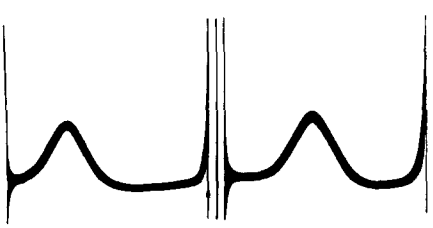


Fig. 8. Sedimentation pattern of purified bacterial carbonic anhydrase in phosphate buffer, pH 6.5, I = 0.05, + 0.10 M NaCl. The experiment was carried out in a Spinco Model E ultracentrifuge at 59 780 rev./min and 20 °C ± 0.1 °C. The initial concentration of protein was 2.9 mg/ml. Photographs of the schlieren patterns were made on Kodak metallographic plates.

TABLE IV

STABILITY OF CARBONIC ANHYDRASE FROM *Neisseria sicca*

Enzyme solution from *Step 2* in the purification procedure was dialyzed against water. Aliquots of this solution were mixed with buffer of different pH and ionic strengths. The solutions were kept in stoppered test tubes at 8–10 °C and aliquots were taken directly after mixing and 2, 6 and 15 days later and assayed for enzymic activity.

pH	*Buffer*	*Ionic strength*	*Percentage of enzymic activity after various times of incubation*			
			0 days	*2 days*	*6 days*	*15 days*
4.5	Sodium acetate	0.05	100	32	25	4
		0.20	100	22	21	5
5.5	Sodium acetate	0.05	100	97	95	80
		0.20	100	101	105	104
6.5	Sodium phosphate	0.05	100	99	99	98
		0.20	100	98	102	93
7.5	Tris–HCl	0.05	100	102	109	106
		0.20	100	101	100	95
8.5	Tris–HCl	0.05	100	103	103	98
		0.20	100	93	94	86

Enzyme stability

Table IV illustrates the stability of the bacterial carbonic anhydrase at different pH. The experiment was carried out with an enzyme solution from *Step 2* in the purification procedure. During the experiment a slight turbidity gradually developed in the solutions but the enzyme activity did not decrease significantly, except at pH 4.5. The partially purified enzyme thus seems to be reasonably stable over a pH range from 5.5 to at least 8.5 at 8–10 °C and moderate ionic strength ($I = 0.05$–0.20). Some evidence has been obtained that the enzyme at higher purity may be less stable than the partially purified preparations. With the enzyme solution from the final purification step a decrease in specific enzyme activity upon standing has been noted which can be restored with a concomitant loss of protein by repeating the SE-Sephadex chromatography *(Step 4)*.

DISCUSSION

The procedure suggested here for the preparation of carbonic anhydrase from *N. sicca*, strain 6021, obviously leads to a considerable purification of the enzyme as judged from the increase in specific enzyme activity (see Table III). The result of the polyacrylamide gel electrophoresis (Fig. 7) and the sedimentation pattern in the ultracentrifuge (Fig. 8) show that the enzyme derived from the final purification step has a high degree of homogeneity. This is further strengthened by the studies of the properties of the purified bacterial carbonic anhydrase described in the following paper[9]. Throughout the purification procedure the bacterial enzyme appears to behave as a single protein species. This is in contrast to the polymorphic patterns frequently encountered in studies of carbonic anhydrase from mammalian erythrocytes[18].

The purpose of the present investigation was to try to evaluate the potential value of the bacterial enzyme for studies on the relationship between structure and function of carbonic anhydrase. A great advantage with bacteria as the starting ma-

terial compared with the hitherto used mammalian sources[1] would be the possibility of varying the protein structure by genetic alterations of the organism. However, a limitation to the usefulness of the enzyme from *N. sicca* described here is the low yield of purified enzyme that at present appears to put restrictions on the possibilities for detailed structural studies and other investigations requiring large amounts of material. The present paper describes the development of a growth medium which increases the yield by a factor of about three. Even under these improved conditions a bacterial culture of 20 l gives only 15–25 mg of purified enzyme. The recovery of enzyme in the various steps of the purification procedure is fairly satisfactory (see Table III) and this low yield of purified enzyme probably reflects a low level of biosynthesis. It should be pointed out, however, that the growth conditions described here are, possibly, still far from optimal with respect to enzyme synthesis and it is conceivable that a more extensive study of growth conditions could considerably improve the yield of enzyme.

Several investigators[19,20] have shown that the growth of various *Neisseria* species can be inhibited by the presence of low concentrations of carbonic anhydrase inhibitors of the sulfonamide type in the growth medium. This effect has been reported to be restricted to strains containing carbonic anhydrase activity[10] which strongly suggests that the presence of this enzymic activity in the bacterial cell is essential for growth. The bacteriostatic action of the enzyme inhibitors is counteracted by an increase in the concentration of carbon dioxide[20,10]. The explanation suggested is that the presence of enzymic activity is not an indispensable requirement at high concentrations of CO_2 or bicarbonate, rather than assuming a competition between sulfonamide and CO_2 or bicarbonate (*cf.* refs 21 and 22). The former explanation is supported by the observation made here that at an increased concentration of CO_2 in the growth medium the bacterial cells contain only very small amounts of carbonic anhydrase. This effect, probably caused by a repressive action of CO_2 or bicarbonate on carbonic anhydrase synthesis, has also been observed for bacterial cells grown on agar. It is interesting to note that a similar pattern for regulation of carbonic anhydrase synthesis appears to occur in green plants as reported for unicellular algae[23,24]. In plant cells carrying out the fixation of carbon dioxide according to the Calvin cycle of photosynthesis the enzyme is completely or to a major extent located in the chloroplasts[25,26]. Obviously, the chloroplast carbonic anhydrase is of importance for CO_2 fixation[27,24] but how the enzyme contributes in detail is not yet known. A functional parallelism between the algal and bacterial systems would be that the enzyme by catalysis of the reversible hydration of carbon dioxide plays the role of facilitating the access of CO_2 or bicarbonate at sites in the cell where it is required and consumed in metabolic reactions. At higher concentrations of CO_2 or bicarbonate simple diffusion and the rate of the uncatalyzed interconversion between CO_2 and bicarbonate may sufficiently cover the metabolic requirements and the synthesis of the enzyme can be repressed without harmful effects. While in algae the Calvin carboxylation cycle may be the pertinent reaction, the situation in *Neisseria* is more unclear. It is, however, well known that CO_2 can act as a metabolite in *Neisseria*. The energy metabolism in *N. meningitidis* has been shown to be strongly influenced by CO_2 or bicarbonate[28] and the carboxylation of phosphoenolpyruvate to oxaloacetate has been proposed as a key reaction in the metabolism of this organism[29].

The bacteriostatic action by carbonic anhydrase inhibitors of the sulfonamide

type described for several *Neisseria* species[19,10] has also been found for the particular strain studied here and may be utilized in constructing selective media for obtaining structural variants of carbonic anhydrase. Results with other microorganisms have shown that one possible mechanism to achieve resistance towards an enzyme inhibitor or substrate analog would be structural changes of the enzyme molecule[30,31]. Structural alterations selected for in the presence of an enzyme inhibitor would be those decreasing the binding strength of the inhibitory compound or increasing substrate binding or turnover rate. Mutational changes elsewhere in the enzyme molecule without effect on these properties will not be selected for. This means that the isolation of resistant mutants may be expected to become a technique to vary structurally the part(s) of an enzyme molecule of special interest from an enzymological point of view.

Structural studies by X-ray diffraction on one of the forms of human carbonic anhydrase[32,1] show that the active site of the enzyme is located in a crevice of the protein molecule and that sulfonamide inhibitors are bound in this crevice close to the zinc ion which has been ascribed a central role in the catalytic function of the enzyme[1]. It would be conceivable that amino acid side chains affecting the binding of sulfonamides may also be of importance for the catalytic properties of the enzyme. This is supported by the finding for one of the human enzymes that a structural change by chemical modification of a single amino acid side chain in the active site region affects both catalytic properties and sulfonamide binding[33]. Mutants of *Neisseria sicca*, strain 6021, resistant towards carbonic anhydrase inhibitors of the sulfonamide type can be obtained as spontaneous mutations or in larger amounts after chemical mutagenesis with nitrosoguanidine (J. Brundell, K. Jyssum and P. O. Nyman, unpublished results). Such mutants have been collected and are under investigation.

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