From the Department of Physiology, Endocrinology
and Blood Grouping,
Royal Veterinary and Agricultural University, Copenhagen, Denmark.

PURIFICATION AND PROPERTIES
OF THREE PIG
ERYTHROCYTE CARBONIC ANHYDRASES

By

Jørn Hejgaard

HEJGAARD, JØRN: *Purification and properties of three pig erythrocyte carbonic anhydrases.* Acta vet. scand. 1972, *13*, 348—371. — Three distinct forms of the zinc containing enzyme carbonic anhydrase were isolated from pig erythrocytes. One low activity type enzyme and two genetic variants of the high activity type enzyme with identical CO_2 hydratase activities which were 8 times as high were isolated from Danish Black and White Swine. In the isolation procedure described, the hemoglobin was eliminated by precipitation with chloroform-ethanol, and the isoenzymes were separated by DEAE-Sephadex chromatography. A number of enzymatically active minor components were separated. They were apparently all genetically linked to one of the three major components. The three purified isoenzymes behaved as homogeneous components during isoelectric focusing and electrophoresis at different pH values. They were characterized in terms of molecular weight, isoelectric pH, zinc content, amino acid composition, and enzymatic activity against CO_2, p-nitrophenyl acetate, and β-napthyl acetate. The circular dichroism of the enzymes in the ultraviolet region was studied. The properties of the enzymes were similar to those of carbonic anhydrases of corresponding types isolated from other mammalian species. Sulphur containing amino acid residues were absent in the low activity type enzyme. The amino acid composition of the two high activity mutants deviated only in that an arginine residue in the most widespread genetic variant was replaced by a histidine residue in the less frequent variant. Otherwise the two mutants showed identical properties.

carbonic anhydrase; pig; erythrocyte; isoenzymes; genetic variants; DEAE-Sephadex; isoelectric pH; amino acid composition; CO_2 hydratase activity; esterase activity; circular dichroism.

Multiple molecular forms of the enzyme carbonic anhydrase have been isolated from red cells of many mammalian species including man, rhesus monkey, cow, horse, dog, guinea pig, rab-

bit and rat. The results of numerous studies of the chemical, physical, and enzymatic properties of the carbonic anhdrase isoenzymes and of their physiological role have been reviewed by *Edsall* (1968) and *Maren* (1967).

Carbonic anhydrases so far isolated can be classified into two chemically distinct enzyme types. Owing to their highly different enzymatic activity they have been designated "high activity type" and "low activity type" enzymes, respectively. Normally both molecular forms are present in the red cells, but only one type has been demonstrated in cattle. Presumably one type of the enzyme arose from the other by a process of gene duplication. Studies of isoenzymes in primates have shown that the two types of enzyme are under separate genetic control (*Tashian* 1969). Genetic variants of one or both types were observed in many species including man and other primates (*Tashian*), cattle (*Sartore & Bernoco* 1966), horse (*Sandberg* 1968), and sheep (*Tucker et al.* 1967). Only the rarely encountered variants of the human low activity enzyme have been studied to some extent. The observed polymorphisms are apparently all regulated by codominant autosomal allelic genes. A number of enzymatically active minor components encountered in apparently all species are undoubtedly conformational isomers which do not represent additional genetic loci (*Tashian; Funakoshi & Deutsch* 1969).

A study of the pig erythrocyte carbonic anhydrases was initiated by the demonstration in this laboratory of two genetic variants of the high activity enzyme in Danish Black and White Swine (*Kloster et al.* 1970). The frequency of the least widespread type was 20 % (homozygote plus heterozygote). One low activity type of enzyme was present in all examined animals.

The three isoenzymes were isolated in an apparently homogeneous form. Some physical and chemical properties of the two types of the enzyme and their genetic variants in this species were characterized to form the basis of studies of the distribution and function of the isoenzymes in pig tissues other than blood. It was also of interest to compare with carbonic anhydrases from other mammalian species at an equal evolutionary stage.

MATERIALS AND METHODS

Chemicals. The following reagents were used: Sephadex G-75 superfine and DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden), acetazolamide (K & K Laboratories, Hollywood, Calif., USA); oval-

bumin, bovine serum albumin (fract. V), p-nitrophenyl acetate and β-naphthyl acetate (Sigma, St. Louis, Mo., USA); porcine trypsin (Novo, Copenhagen, Denmark); Fast Blue BB Salt (diazotized 4-benzoylamino-2,5-dimethoxyaniline, $ZnCl_2$) (Gurr, London S.W. 14, England); Ampholine, pH 3—10 and pH 5—8 (LKB-Produkter, Bromma 1, Sweden). Danish potato starch (Struers, Copenhagen, Denmark) was hydrolyzed according to *Smithies* (1955). Most other chemicals were of analytical reagent grade.

Preparation of chloroform-ethanol extract. Freshly obtained blood was used in all experiments. The erythrocytes were isolated by centrifugation, and after removal of the supernatant and the leucocyte layer they were washed three times with 0.9 % NaCl. The packed cells were hemolyzed by addition of an equal volume of water and dialyzed overnight at 4°C. The extraction procedure applied was essentially that of *Armstrong et al.* (1966). After the removal of the denatured hemoglobin by centrifugation the supernatant was filtered and dialyzed at 4°C.

Concentration of protein solution. The extract was concentrated by placing the dialysis tubes in solid $(NH_4)_2SO_4$. This procedure reduced the volume of the enzyme extract to one fifth of its original volume in less than two hrs. The tube was then transferred to a saturated $(NH_4)_2SO_4$ solution adjusted to pH 7.0 with solid Tris and left for 12 hrs. at 4°C. The precipitated protein was isolated by centrifugation, and the precipitate was stored at 0°C.

DEAE-Sephadex chromatography. Separation by column chromatography was in all cases performed at 7—8°C. Elutions were carried out with 0.045—0.2 M Tris adjusted with HCl to pH 8.2 at 25°C. The flow rate was 25—30 ml per hr., and fractions of 10.5 or 13.5 ml were collected. DEAE-Sephadex A-50 was pretreated according to the manufacturer's directions and the suspension was equilibrated with start buffer in a 25 × 500 mm column. The protein concentrate was dialyzed against the 0.045 M start buffer, filtered, and applied to the top of the column. The low activity carbonic anhydrase was eluted with the start buffer, while the two other enzymes together with all the minor components were eluted by stepwise increase of the ionic strength of the buffer. The concentration and storage of the isolated fractions were performed according to the procedure described above. Some fractions were lyophilized for amino acid analysis, determination of nitrogen, etc.

Assay of CO_2 hydratase activity. In the course of the fractionations, the CO_2 hydratase activity was determined by a modification of the method of *Wilbur & Anderson* (1948). The incubation mixture was 1 ml of 0.025 M veronal buffer, pH 8.2, containing 1 % (w/v) Bromthymol Blue and 200 μM EDTA. To this was added 0.1 ml of enzyme solution in 0.01 % peptone, and the solution was equilibrated at 0.5°C. Then 1 ml of H_2O saturated with CO_2 at the same temperature was injected. The time interval from injection until colour change from a yellowish-green to pure yellow was recorded and the specific

activity calculated as described by *Rickli et al.* (1964). Conditions were so selected that the non-catalyzed reaction lasted 100—110 sec. and the catalyzed reaction 18—25 sec. Within this range, results were reproducible and the activity varied linearly with protein concentration.

Assay of esterase activity towards p-nitrophenyl acetate. The buffer, 2.4 ml of 0.0125 M Tris-maleate adjusted to pH 7.15 with NaOH, was equilibrated in a 1 cm cuvette at 30°C. Then 0.3 ml of a 12.5 mM solution of p-nitrophenyl acetate in 40 % (v/v) acetonitrile or in 50 % acetone was added. Absorbance at 400 nm was followed in a recording spectrophotometer through 9 min., using 0.01 M buffer as reference. After incubation for 2 min., 0.3 ml enzyme solution was added. After further 5 min., 0.1 ml of 4 mM acetazolamide was added, upon which the rate of increase in absorbance dropped to the non-enzymic level. The esterase activity was determined as the difference between rates of hydrolysis after and before enzyme had been added. The readings before addition of enzyme and after addition of acetazolamide provided an internal blank and enabled a correction for contributions from other esterases present in impure preparations. In kinetic assays the substrate concentration was varied in the range 0.2—2 mM.

Assay of esterase activity towards β-naphthyl acetate. An enzyme solution in 1.6 ml of water was added to 0.2 ml of 0.1 M Tris-maleate buffer adjusted to pH 7.15 with NaOH, and the mixture was equilibrated at 30°C. Subsequently, 0.2 ml of a 12.5 mM β-naphthyl acetate in 40 % (v/v) acetonitrile or in 50 % acetone was added, and the mixture was incubated for 20 min. at 30°C. The reaction was stopped by addition of 0.5 ml of 4 mM acetazolamide. Immediately after 0.5 ml of a 0.4 % solution of Fast Blue BB Salt was added. The colour was developed for 40 min. at room temperature. Upon thorough mixing, 1 ml was withdrawn and diluted with 3 ml of 96 % ethanol. The absorbance was measured at 550 nm against a reagent blank.

Starch gel electrophoresis was carried out according to the method of *Smithies*. The concentration of starch was 12 %. The gel and vessel buffer was 0.05 M Tris, adjusted with glycine to pH 8.8 and 8.4, respectively. A buffer system described by *Kloster et al.* (1970) was used in some experiments. Electrophoresis was carried out in a horizontal, water-cooled apparatus at 18 v/cm. The separated proteins were localized with Amido Black. Staining for esterase activity was carried out by placing the gel in an aqueous solution of 0.02 % β-naphthyl acetate, 0.2 % Fast Blue BB Salt, 5 % ethanol at 37°C for 3 hrs. Zones representing CO_2 hydratase activity were in some experiments localized by means of Bromthymol Blue and CO_2 (*Tashian* 1969).

Isoelectric focusing was performed in a 110 ml capacity apparatus (LKB-Produkter, Bromma 1, Sweden) as described by *Vesterberg & Svensson* 1966). The column contained 0.75 % (w/v) Ampholine pH 5—8, and 0.25 % Ampholine pH 3—10. It was stabilized against convection by a 46—0 % (w/v) continuous sucrose gradient. The voltage

was gradually increased from 200 to 400 v. The temperature of the cooling water was 10°C. When equilibrium was reached, fractions of 1.25 ml were collected and their pH determined immediately using a combination electrode connected to a Model 27 pH-meter (Radiometer, Copenhagen, Denmark). Protein content and enzyme activity were determined as in fractions isolated by column chromatography.

Purity of enzyme fractions was controlled by isoelectric focusing in polyacrylamide gels (*Wrigley* 1968) using 6 × 125 mm tubes. Chemical polymerization was used for the preparation of 6.5 % gels containing 2 % (w/v) of a mixture of equal volumes of Ampholine pH 3—10 and pH 5—8. Separation was done at 2 mA per tube until the voltage had risen to 300 v, at which level it was kept constant. Proteins were fixed in 12.5 % trichloroacetic acid and stained with Coomassie Blue (*Chrambach et al.* 1967).

Determination of molecular weight by gel filtration was carried out according to the method of *Andrews* (1964). Sephadex G-75 superfine was packed in a 1 × 120 cm column. The proteins were eluted at 2°C with 0.025 M phosphate buffer, pH 7.0, containing 0.25 M-KCl. The column was calibrated with bovine serum albumin, ovalbumin, and porcine trypsin. The elution volumes were determined by monitoring the effluent at 280 nm and, in the case of carbonic anhydrases, by assay of the CO_2 hydratase activity. A Blue Dextran reference was included in all experiments.

Amino acid analyses were performed in accordance with *Spackman et al.* (1958) using a Beckman 120 C amino acid analyzer provided with an Infotronics two-channel integrator. Periods of hydrolysis were 16, 24, and 48 hrs. The amounts of NH_3, serine, and threonine were determined by extrapolation to zero time. The values obtained after 48 hrs.' hydrolysis were used for valine and isoleucine.

Tryptophan was determined using the same procedure except that the acid standard hydrolysis was supplied with 3 % (v/v) thioglycolic acid (*Matsubara & Sasaki* 1969). The samples were hydrolyzed for 24 hrs. Tryptophan values were calculated relative to contents of arginine and lysine, assuming 83 % recovery. In some control analyses of lysozymes from man and egg white, the recovery varied between 81 and 84 %. Tyrosine and tryptophan were also determined spectrophotometrically according to *Goodwin & Morton* (1946).

Half-cystine was determined as cysteic acid (*Spencer & Wold* 1969) using 24 hrs. hydrolysis in the presence of 2.5 % (v/v) dimethyl sulphoxide. Upon amino acid analysis, the content of half-cystine was calculated relative to aspartic acid, using the integration constant of the latter for quantitation of both amino acids. This method gave a 100 % recovery by analysis of human lysozyme and glutathione.

Determination of zinc. Enzyme solutions were dialyzed for 48 hrs. against three shifts of Zn-free water. The protein was precipitated in boiling 5 % TCA. Upon centrifugation, the zinc in the supernatant was determined with an EEL Model 140 atomic absorption spectrophotometer using the third outer dialysate as reference.

Determination of nitrogen. The micro Kjeldahl method of *Parnas* (1938) was employed.

Determination of protein. Absorbance at 280 nm served as a measure of protein concentration, taking $A_{280}^{1\%}$ as 18.6 for enzyme Ia and 18.8 for IIb and IIa. These values were calculated on the basis of amino acid analyses of enzyme solutions as described by *Rickli et al.* Calculations were made on the assumption that 1 mole of protein equalled 39 moles of basic amino acids (Lys + His + Arg) in all three proteins. The molecular weights used are recorded at the bottom of Table 2.

Circular dichroism was determined on a Roussel-Jouan Dichrograph CD 185. The spectra in the range 300—240 nm were recorded using a 1 cm cell and in the 240—200 nm range using a cell with 0.2 cm light path. The Dichrograph was operated at maximal sensitivity. Mean residue molecular weights obtained from the amino acid analysis data were used in calculating the mean residue molecular ellipticity (θ).

RESULTS

Nomenclature. The nomenclature used by *Tashian* (1969) in his classification of primate carbonic anhydrases was preferred to the generally accepted B/C-nomenclature (*Rickli et al.* 1964, *Furth* 1968). *Tashian*'s nomenclature provides a distinct division in two enzyme types and it permits an unambiguous subclassification of the genetic variants of the two types and their conformer minor components. The low activity type enzyme isolated was designated Ia, the two high activity mutants designated IIa and IIb, respectively, in conformity with the A/B-nomenclature used by *Kloster et al.* (1970). Peaks from ion exchange or electrophoretic bands representing conformational isomer variants of the three major components, or apparently well-defined enzyme aggregates, are designated + or —, according as the mobility in DEAE-Sephadex, or electrophoretically towards the anode, is higher or lower than that of the corresponding major component (e.g., Ia$^+$, IIb$^-$).

Preparation of carbonic anhydrase isoenzymes. Extraction with chloroform-ethanol resulted in an almost 100 % precipitation of the hemoglobin. The extracts were of a yellowish colour, and the protein precipitated with $(NH_4)_2SO_4$ was pale greyish. More than 65 % of the enzyme activity of the hemolysate was recovered in this concentrate. No further activity could be iso-

lated from the hemoglobin paste. The carbonic anhydrases could also be separated from the hemoglobin by DEAE-Sephadex chromatography of the dialyzed hemolysate (*Armstrong et al.* 1966). Attempts to isolate the hemoglobin by precipitation with $(NH_4)_2SO_4$ (*Furth*) gave unsatisfactory results.

The three isoenzymes were separated by DEAE-Sephadex chromatography in a single run. The low activity enzyme Ia and the high activity type IIb were isolated from hemolysates from animals which, regarding the high activity type enzyme, were homozygous. The IIa form was isolated together with Ia and IIb from blood derived from heterozygotes. Separation was optimal when Ia was eluted with 0.05 M, IIb with 0.1 M, and IIa with 0.15 M buffers. Gradient elution always resulted in an incomplete separation of major and minor components. Fig. 1 shows a typical result of DEAE-Sephadex chromatography. For clarity, only the hydratase activity of the three major components in the extract is recorded. Several other protein peaks were observed, some of which showed CO_2 hydratase activity.
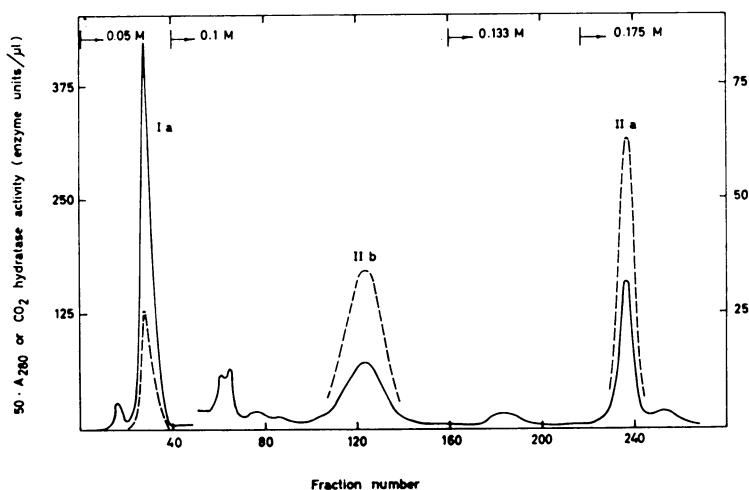

F i g u r e 1. Elution of Ia, IIb, and IIa isoenzymes from DEAE-Sephadex. (————) absorbance at 280 nm; (- - - - -) CO_2 hydratase activity. The enzymes were eluted with Tris-HCl pH 8.2 (25°C), Ia with 0.05 M, IIb with 0.1 M, and IIa with 0.175 M buffer as indicated at the top of the figure. The left ordinate scale applies to fractions 1—43, while the remaining fractions refer to the right hand scale. Absorbance was measured after appropriate dilution. Volumes of the collected fractions were 13.5 ml.

Electrophoresis of chloroform-ethanol extracts from larger numbers of pigs revealed the presence of a considerable number of active minor components. Animals of all three high activity enzyme phenotypes were studied. Both of the major high activity components were always associated with at least 4 active minor bands which, undoubtedly, are products of the same genetic locus. The bands were located in identical positions relative to the major bands, and corresponding bands probably represent the same conformational changes in their respective major forms. Together with the low activity enzyme band Ia, two very weak bands with mobilities between those of Ia and IIb could be demonstrated in all extracts. These bands are probably analogous variants of the Ia enzyme.

This feature is illustrated by the results obtained by DEAE-Sephadex chromatography of a chloroform-ethanol extract with an atypically high content of minor components (Fig. 2). The extract was derived from a homozygous animal (type IIb). Three protein peaks without CO_2 hydratase activity were first eluted. After elution of the low activity enzyme (Ia), two diminutive peaks with low activity (Ia$^+$) appeared. Then at least three peaks with high activity (IIb$^-$) followed, prior to elution of the major component IIb. Finally, one or two high activity carbonic anhydrases were eluted (IIb$^+$) with 0.2 M buffer. It is obvious that
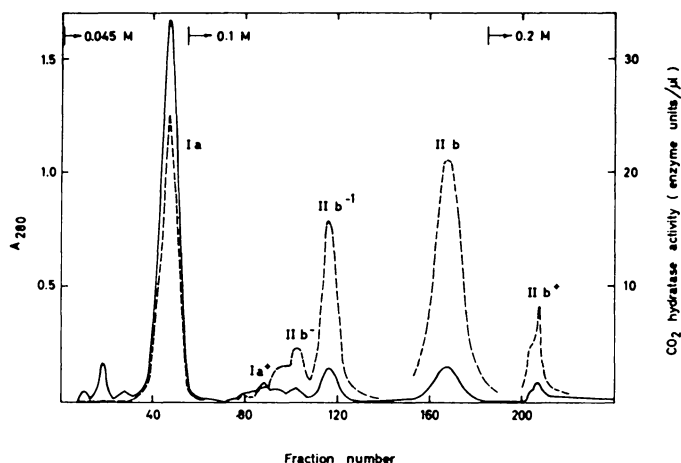

F i g u r e 2. Elution of conformere Ia and IIb isoenzymes from DEAE-Sephadex. (————) absorbance at 280 nm; (-----) CO_2 hydratase activity. Concentrations of elution buffers are indicated at the top of the figure. 10.5 ml fractions were collected.

the high activity enzyme is by far the most labile type. In this extract, the minor components represented more than 60 % of the high activity protein. The CO_2 hydratase activity of the minor components was always lower than that of the major component.

The minor form present in greatest quantity was designated IIb^{-1}. This component could be identified in chloroform-ethanol extracts as a well-defined chromatographic peak, and in electrophoresis it migrated apparently as one band. A complete purification was not attempted. One fraction collected from the central part of the IIb^{-1} peak (Fig. 2) was examined. The CO_2 hydratase activity and the esterase activity were 75—80 % of that of the IIb component. The fraction was concentrated by $(NH_4)_2SO_4$ and lyophilized. Electrophoresis of this product showed that more than 80 % of the protein was by now present in the IIb band, the remaining percentage being localized in two enzymatically active, weak bands of lower anodic mobility. The mobility of the IIb and IIa forms were not changed by lyophilization. It may thus be assumed that the IIb^{-1} peak (Fig. 2) represents a IIb variant, generated by a reversible conformational change. A corresponding IIa^{-1} form was observed. The intensity of the secondary bands varied much from extract to extract, depending on minor variations in the procedure. In some experiments where extremely high concentrations of chloroform-ethanol were used for the extraction, a subsequent electrophoresis showed that IIb^{-1} and IIa^{-1} were the predominating high activity enzyme bands present. Staining with Bromthymol Blue, however, revealed the presence of the high activity minor bands also in crude hemolysates. Thus, they are to some extent present in vivo or might be formed during electrophoretic separation.

Table 1 gives typical examples of composition of chloroform-ethanol extracts, as calculated after DEAE-Sephadex chromato-

T a b l e 1. Separation of crude carbonic anhydrase preparations in genetic variants by DEAE-Sephadex chromatography.

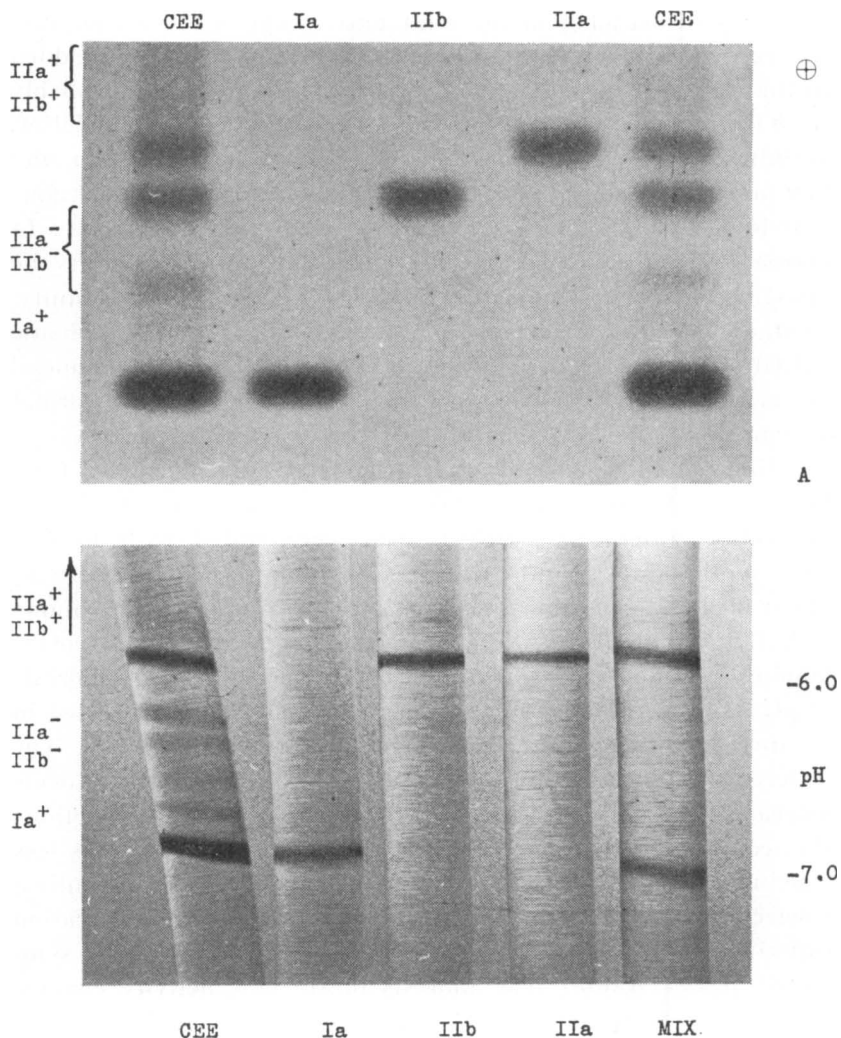|	II phenotype	Carbonic anhydrase		
		Ia	IIb	IIa
Per cent of active protein	bb	76	24	—
	ab	77.5	11.5	11
Per cent of CO_2 hydratase activity	bb	28	72	—
	ab	29.5	36	34.5

F i g u r e 3. Starch gel electrophoresis (top) and isoelectric focusing (bottom) of the fractions isolated after rechromatography on DEAE-Sephadex. Approx. 80 µg of the isolated isoenzymes were applied. (CEE) chloroform-ethanol extract; (MIX) mixture of the three iso-enzymes; (A) application of sample; ($\oplus$) anode. Some minor components in the chloroform-ethanol extract are indicated in the left side of the figure. To the right is shown an approximate pH scale applying to the isoelectric focusing.

graphy. At least 90 % of the total activity applied to the column was recovered. Contributions from minor components were added to the value of the major component from which they probably were formed and ascribed the same specific activity as the latter. Within experimental error, the distribution between high and low activity carbonic anhydrases was identical in heterozygous individuals and homozygous animals of the examined type. In extracts of blood from heterozygotes the ratio between the amounts of the two high activity enzymes was very close to unity. In studies of genetic variants of the human low activity carbonic anhydrase, *Tashian* observed that the three rare mutants included in his study were produced in lower quantities than the normal enzyme.

Minor components and other proteins, if any, in pooled fractions from the major peaks from several runs were eliminated by rechromatography. The proteins were eluted with buffers with lower ionic strength than previously applied, but otherwise under identical conditions. Approximately 5 times the bed volume of buffer was used. All three isoenzymes appeared in fully overlapping, symmetrical protein and activity peaks. Electrophoresis at pH 7 and 8.8 of fractions from rechromatography revealed in all three cases apparently homogeneous proteins (Fig. 3). The difference in electrophoretic mobility of the IIb and IIa forms increased with pH. After isoelectric focusing, the three fractions stained as distinct single bands. This criterium, however, is less conclusive, since the two high activity enzymes have identical isoelectric pH, as illustrated in Fig. 3. In Sephadex gel filtration experiments the three preparations were eluted as single, symmetric peaks. Amino acid analysis of the low activity enzyme showed that cysteine and methionine were absent in this protein. The total absence of these two peaks in the chromatograms confirms the purity of the isolated Ia protein.

Properties of the isoenzymes

Ultraviolet absorption. The molar extinction coefficient ε_M at 280 nm, which was used as standard measure of protein concentration, was calculated on the basis of the amino acid analyses as described under "Methods". The values obtained were 53,700 M^{-1} cm^{-1} for Ia and 54,000 M^{-1} cm^{-1} for IIb and IIa. ε_M was also calculated after Kjeldahl nitrogen analyses of protein solutions

with measured absorbance. Nitrogen percentages calculated from
the amino acid analyses were used. The values obtained by this
method were 2—3 % lower than those presented above. The
reason may be that the amide content in the proteins was over-
estimated. The uncertainty involved in the amide determination
was marked, and the values recorded in Table 2 are higher
than those observed in carbonic anhydrases from other species.
Extinction coefficients may also be obtained by summation of
contributions from the individual tyrosine and tryptophan resi-
dues. Values calculated in this way were in all three proteins
13 % lower than those observed. This discrepancy is of the same
magnitude as observed in a series of other proteins (*Wetlaufer*
1962).

Molecular weight. Gel filtration of the three isoenzymes re-
sulted in all experiments in molecular weights ranging between
30,000 and 30,500. Thus, molecular weights of the three carbonic
anhydrases are assumed to be nearly identical. It is recognized
that the elution volumes are more directly determined by Stokes'
radii, and the absolute value obtained is probably more uncertain.

Zinc content. Zinc analyses of the three enzymes gave for Ia,
0.227 %; for IIb, 0.228 %; and for IIa, 0.235 %. These results
represent the average of three analyses, and the maximal devia-
tion was less than ± 0.010 %. On the assumption that each mole-
cule contains one Zn atom, the results indicate that the molecular
weights of the three proteins are close to 29,000.

The amino acid composition of the three isolated isoenzymes
is presented in Table 2. Initially the composition was calculated
on the assumption that all three proteins contain 260 residues
per molecule. Optimal adjustment to integral figures in the range
245—270 residues per molecule was obtained for Ia when the
value of aspartic acid was assumed to be exactly 33.0 residues
per molecule. For both high activity enzymes this was achieved,
assuming 29.0 glutamic acid residues per molecule. The results
obtained in this way are recorded to the left in the table. The
values were then normalized to the nearest integer. The calculated
molecular weights were for all three proteins found to be close
to 28,800, assuming one Zn atom per molecule. They are well
within the range found by Zn determination and gel filtration.

Tyrosine and tryptophan were also determined spectrophoto-
metrically. When this method is applied, it is essential to await

Table 2. Amino acid composition of pig carbonic anhydrase Ia, IIb and IIa.

The calculated molecular weights equal the sum of the amino acid residues plus 1 zinc plus 1 H_2O. The nitrogen recovery was calculated on the basis of Kjeldahl analysis of the sample used for amino acid analysis.

| | Residues per molecule | | | | | |
| | found | | | to nearest integer | | |
	Ia	IIb	IIa	Ia	IIb	IIa
Lysine	21.2	17.6	17.9	21	18	18
Histidine	12.9	12.1	13.3	13	*12*	*13*
Arginine	4.9	8.6	7.6	5	*9*	*8*
Amide NH_3	(33)	(35)	(35)	(33)	(35)	(35)
Aspartic acid	33.0	27.3	27.2	33	27	27
Glutamic acid	20.3	29.0	29.0	20	29	29
Glycine	17.7	22.8	23.1	18	23	23
Alanine	19.4	12.0	12.0	19	12	12
Valine	15.0	15.7	15.6	15	16	16
Leucine	22.0	21.3	21.0	22	21	21
Isoleucine	15.7	9.5	9.5	16	10	10
Proline[a]	17.0	17.5	17.5	17	17	17
Serine	26.8	19.3	18.9	27	19	19
Threonine	8.7	12.9	12.9	9	13	13
Half-cystine[b]	0.0	2.1	2.1	0	2	2
Methionine	0.0	2.6	2.4	0	3	3
Phenylalanine	9.0	11.5	11.6	9	12	12
Tyrosine	10.2	6.0	6.0	10	6	6
Tryptophan[c]	5.8	7.0	7.0	6	7	7
Total no. of residues				260	256	256
Nitrogen recovered, %				99	101	98
Calculated molecular weight				28855	28802	28783

[a]) The observed yields were expected to be slightly high when cysteine is present.

[b]) Determined as cysteic acid by the method of *Spencer & Wold* (1969).

[c]) Determined by the method of *Matsubara & Sasaki* (1969).

a complete ionization of the tyrosine residues (*Beaven & Holiday* 1952). The low activity carbonic anhydrase was apparently unfolded after a few min. at pH 13. Absorption spectra of solutions of the two high activity type enzymes in 0.1 M-NaOH changed steadily during three hrs. at room temperature after which the absorbance at 280 and 294.4 nm became constant.

Absorption measurements after alkaline denaturation for 3½ hrs. gave tryptophan values in fair agreement with those obtained by amino acid analyzer, namely 5.8, 7.1, and 7.2, residues per molecule of Ia, IIb, and IIa, respectively. The observed tyrosine values, however, were higher than those obtained by the amino acid analyzer, and the ratio tyrosine to tryptophan was 2.10 for Ia and 1.25 for the two high activity enzymes. If these values were used with the tyrosine values obtained by amino acid analyses for a calculation of the tryptophan content, only 5 residues should be present per molecule of all three proteins. A tryptophan content of this magnitude can hardly be in agreement with the observed molar extinction coefficients. The reason for this discrepancy may be the protracted equilibration period preceding the photometry.

An interesting feature of the amino acid analyses is that sulphur is completely absent in the low activity type enzyme. All carbonic anhydrases so far analysed have been found to contain at least one methionine residue. Absence of cysteine was observed only in the high activity bovine enzyme (cf. *Furth*).
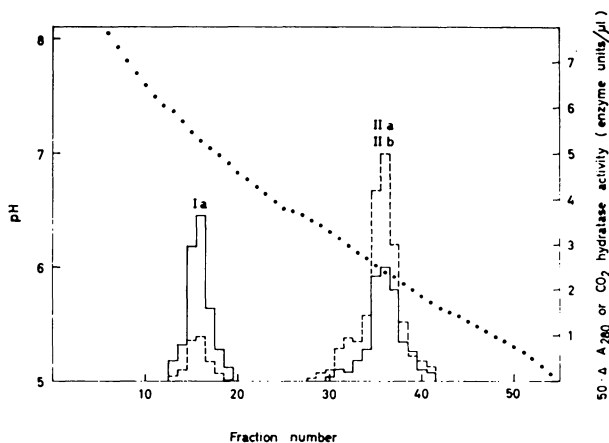

Fraction number

F i g u r e 4. Isoelectric focusing of a mixture of isoenzymes from ion exchange chromatography. Ia (approx. 220 µg); IIb (approx. 65 µg); IIa (approx. 80 µg). (————) Δ A_{280} (absorbance of effluent fraction minus calculated contributions from carrier ampholytes); (-----) CO_2 hydratase activity; (●) pH. Absorbance at 280 nm was measured directly. As the Ampholine represents a relatively large proportion of the measured values, Δ A_{280} provides only an approximate expression of the protein concentration. Neither protein nor enzymatic activity were demonstrable outside the depicted pH-range. The collected fractions were 1.25 ml.

Within the experimental error, the amino acid analyses of the two high activity carbonic anhydrases resulted in exactly the same values for all the amino acids except two. IIb was found to contain 12 histidine and 9 arginine, while the more rare mutant IIa contained 13 histidine and 8 arginine. This difference was substantiated by analyses of various enzyme preparations. As to the other amino acids the difference was maximally 2 % of the observed number of residues, or maximally 0.3 residues.

Isoelectric pH values were determined by focusing of small amounts of the three proteins in stable Ampholine gradients, as illustrated in Fig. 4. The isoelectric pH values thus obtained were 7.15 for Ia and 5.97 for IIb and IIa. The maximum deviation from these values was ± 0.05 pH units. As to the two high activity enzymes it proved impossible to demonstrate any difference in isoelectric pH (cf. also Fig. 3). The enzymatic activity of all three proteins remained unchanged after isoelectric focusing.

Enzymatic activity. Table 3 shows the enzymatic activities of three isoenzyme preparations towards the substrates CO_2, p-nitrophenyl acetate, and β-naphthyl acetate. In accordance with findings in other species, the catalytic properties of the two types of enzyme were highly different, but significant differences in specific activity of the two high activity forms were not demonstrable. When the CO_2 hydratase activity was assayed according to Wilbur & Anderson's method, the type II enzymes were found to be more than 8 times as active as the Ia enzyme. Assays of the relatively weak esterase activity against p-nitrophenyl acetate

T a b l e   3. CO_2 hydratase and esterase activities of pig carbonic anhydrase Ia, IIb and IIa.

The CO_2 hydratase activity is measured in Wilbur-Anderson units (*Rickli et al.* 1964). The units of the esterase activities are μmoles of phenolic compound formed per minute per mg protein. The esterase assays were performed in 4 % (v/v) acetonitrile or 5 % acetone.

Carbonic anhydrase	CO₂ hydratase activity	Esterase activity			
		p-nitrophenyl acetate		β-naphtyl acetate	
		acetonitrile	acetone	acetonitrile	acetone
Ia	29 000	0.15	0.13	0.133	0.095
IIb	240 000	1.41	1.54	0.0165	0.0178
IIa	245 000	1.50	1.56	0.0169	0.0181

showed an activity ratio of the same order of magnitude. With β-naphthyl acetate as substrate, the picture was reversed, and the catalytic capacity of the low activity enzyme Ia was the higher one.

In order to reproduce the CO_2 hydratase assays it was essential to supply EDTA to the mixture. Type II enzymes, which were present in very low concentrations when assayed, gave very fluctuating specific activities when EDTA was not added. It is assumed that the enzymatic activity is affected by traces of metal ions that may be present in the assay mixture or become bound to the protein during isolation. When the activity of type II enzymes was assayed in the presence of 1.5 µM Cu^{++}, but in the absence of EDTA, a 80 % inhibition was observed. A Cu^{++} inhibition of the same relative magnitude has been found with the human high activity enzyme (*Magid* 1967). The activity towards β-naphthyl acetate depended, as apparent from the table, on the solvent used. If assayed in 4 % acetonitrile or in 5 % ethanol, the specific activity ratio Ia to II was 8, while it was approx. 5 in 5 % acetone. The solvent effect on the p-nitrophenyl acetate esterase assays was less pronounced. None of the enzymes were saturated with substrate at the maximum attainable concentration of p-nitrophenyl acetate. The initial velocity of the reaction was almost proportional to the substrate concentration under these conditions, and no satisfactory determination of kinetic constants could be made. Similar results have been obtained with enzymes from other species (*McIntosh* 1969, 1970).

All activity of the three enzyme preparations was completely inhibited by 0.2 mM acetazolamide. Thus, the enzymes did not contain demonstrable amounts of other esterases which are not affected by this potent carbonic anhydrase inhibitor.

Circular dichroism (CD). Carbonic anhydrases produce complex, but characteristic CD spectra at wave lengths in the range 210—300 nm. Spectra of low and high activity enzymes isolated from human erythrocytes presented considerable differences, particularly in the range 260—220 nm (*Beychok et al.* 1966). Fig. 5 shows CD spectra of the three isolated pig enzymes in native form at pH 7. Within experimental error the two high activity carbonic anhydrases IIa and IIb produced completely identical spectra. The CD spectra of corresponding high and low activity enzymes derived from man and pig contained almost
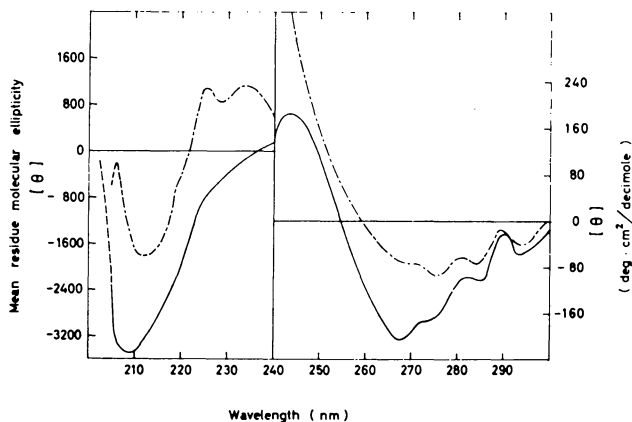
Figure 5. CD spectra of carbonic anhydrase isoenzymes in the range 300—200 nm in 0.025 M phosphate buffer, pH 7.0. (— · — · —) Ia; (————) IIb and IIa. The dotted parts are subject to great uncertainty owing to the high noise level. The left ordinate applies to the 200—240 nm range. The scale to the right applies to the 240—300 nm range.

the same characteristic major bands, but the spectra presented distinct differences in the finer details.

The two high activity enzymes produced a negative extreme at 268 nm. The spectrum became positive at 255 nm, and at 244 nm a distinct maximum was found. In the range from 236 to 205 nm, or below, the spectrum was negative with a maximal negative ellipticity near 209 nm. Similar to this, the low activity enzyme presented a negative band centered close to 275 nm, a very broad positive band with maximum ellipticity at 234 nm, and a minimum at approx. 212 nm. The finer structures of the two spectra showed many similarities. In the range 300—280 nm, small negative extremes were demonstrable at 293 nm and at 285 nm, and maxima were observed at 289 nm and 281—2 nm, and at 272 nm in the II spectra. After passage of the minimum, the Ia spectrum showed a shoulder at 270 nm, and in some spectra a slight shoulder was observed at 250 nm. At wavelengths lower than the extreme positive maximum, a well-defined positive band was seen in the Ia spectrum at 225 nm and, at approximately the same wavelength, a distinct shoulder was present in the II spectrum. Finally, spectra of the low activity enzyme presented a shoulder at 220 nm, and some spectra of the IIb enzyme indicated a shoulder at 218 nm. Below 208 nm spectra were re-

corded at a very high noise level, and, consequently, the dotted part of the curves should be taken with reservation. Spectra of lyophilized enzyme preparations did not deviate significantly from those of native enzymes.

CD spectra are sensitive registrators of conformational changes in protein molecules. The slow denaturation of the Ia and IIb enzymes under moderately acid and alkaline conditions was studied to some extent. After incubation for 3 hrs. at 25°C in 0.025 M formate buffer, pH 4, the finer details of the spectrum of the high activity enzyme remained unchanged in the range 300—245 nm, but the ellipticity in the negative extreme was reduced by approx. 35 %. At lower wavelengths, drastic changes had occurred in the spectrum of the enzyme. The distinct band at 345 nm had extended towards lower wavelengths in a broad, flat peak, and the cross-over point, at 236 nm, at pH 7, had moved downward to 223 nm. Any significant differences were not observed in the vicinity of the minimum point at 209 nm. Changes in the spectrum of the low activity enzyme were not equally marked. The most characteristic finding was a reduced ellipticity at the positive maximum at 225 nm. After incubation for 3 hrs. at pH 4, the two enzyme types thus had almost identical cross-over points from positive to negative ellipticity at approx. 223 nm. After incubation for 90 min. at 25°C in 0.025 M carbonate buffer, pH 10.0, the IIb spectrum was apparently identical with those recorded at pH 7. The spectrum of the low activity enzyme was changing in a way similar to that observed in studies of the corresponding human enzyme at high pH (*Beychok et al.*). Most characteristic was a change to more positive ellipticity values in the range 240—287 nm, while the characteristic, finer structures between 267 and 287 nm apparently remained intact.

DISCUSSION

The general properties of the pig carbonic anhydrases were very similar to those of the corresponding enzymes from other mammalian species. The isoenzymes unambiguously could be classified as low activity type or high activity type enzymes, respectively, on the basis of the structural differences reflected in their amino acid composition, enzymatic activity, and circular dichroism.

As observed in man, monkey, horse, and other species, the

low acticity type enzyme was predominating. The high activity
type seemed to be more labile and, depending on methods used
for isolation, up to 60 % of each of the two high activity enzyme
types could be isolated as minor components, all of which showed
an enzymatic activity lower than that of the corresponding major
enzyme peak. Similar results have been obtained in a study of
the horse high activity enzyme (*Furth* 1968). It has been proposed
that the minor forms could be generated from the major ones
by hydrolysis of amide groups (*Funakoshi & Deutsch* 1969). The
regeneration of the IIb form from the isolated IIb^{-1} fraction
during concentration or lyophilization apparently excludes this
explanation. It seems more probable that the relatively harsh
procedure of lyophilization may have reverted a conformational
change which originally had been produced by the chloroform-
ethanol extraction or by other steps in the isolation procedure.

A comparison with the amino acid composition of the cor-
responding enzymes isolated from man, monkey, cattle, and horse
(data may be obtained from *Furth*) supports the suggestion of
Furth that the higher serine content in the low activity than in
the high activity enzymes is the most characteristic difference
in the composition of the two types. The low activity enzyme of
pigs differs from that of other species so far studied in a rela-
tively high content of isoleucine and the absence of sulphur con-
taining residues. The values obtained do not provide a basis for
extensive taxonomic considerations. The content of lysine in the
high activity enzymes of pigs and in the corresponding enzymes
of cattle and horse is relatively low as compared with the primate
enzymes. Conversely, the contents of alanine and isoleucine,
which almost equal the values from man and monkey, differ
characteristically from those observed in the two other ungulate
species.

The amino acid analyses indicate that one genetic variant of
the high activity enzyme has been produced from the other by
a single amino acid replacement, which can be due to a change
of one nucleotide in the codon. The amino acids involved are
arginine and histidine. The results of the amino acid analyses
seem to be substantiated by the fact that the two proteins ap-
parently have identical isoelectric points below pH 6, where
arginine and most of the histidine are positively charged, and
an increasing difference in relative electrophoretic mobility from
pH 7 to 8, where the histidine residues become more and more

uncharged, while the arginine side chains remain protonized. The residue involved is thus assumed to be at or near the protein surface. The uniform, systematic distribution of the minor components of the two enzymes, as observed by electrophoresis, indicates that this position in the molecule is not involved in processes resulting in a generation of minor components. All other investigated properties of the two high activity enzymes were very similar. Apparently the two mutants were synthesized in identical quantities in the examined heterozygous individuals. Whether the observed difference in composition is the only one cannot be decided until further data are available.

The recorded enzymatic activities show the same characteristic differences between high and low activity enzymes as have been observed in other species. The marked difference in esterase activity towards p-nitrophenyl acetate and β-naphthyl acetate, respectively, seems to be a characteristic feature of the presence of two types of enzymes. In man (*Tashian* 1969), pig, rabbit (*McIntosh* 1970), and rat (*McIntosh* 1969), activities of the high activity enzymes towards p-nitrophenyl acetate ranged very constantly from 5 to 10 times that of the low activity enzymes from the same species. The corresponding ratios between CO_2 hydratase activities were of the same order of magnitude, but varied more depending on the methods applied. In contrast, the low activity type enzyme in the 4 species showed in all cases a higher activity towards β-naphthyl acetate. Also the marked solvent effect on the latter activity of especially Ia indicates differences in the catalytic mechanism for hydrolysis of p-nitrophenyl acetate and β-naphthyl acetate, respectively, of at least one of the enzyme types.

The circular dichroism spectra contain the same major ellipticity bands as those observed in the two corresponding human enzymes. A correlation with ORD studies of carbonic anhydrases of horse (*Furth*) and primates (*Beychok et al.* 1966, *Rosenberg* 1966) confirms that the characteristic difference in structure of the two types of enzyme is reflected in the optical asymetry in their absorption bands. The general features of the very similar human enzyme CD spectra have been discussed in detail by *Beychok et al.* The CD bands in the wavelength range studied are attributable primarily to optically asymmetric interactions of the aromatic amino acids, in particular tyrosine and tryptophan. This may explain the observed differences in the fine struc-

ture patterns of the spectra of the corresponding enzymes from
man and pig, and, correspondingly, the different effect of a mo-
derate raise of pH on the pig enzymes may depict the greater
content of tyrosine residues in the Ia enzyme. A more detailed
discussion of these features seems rather futile until more in-
formation of the three-dimensional structures of the two types
of enzymes is available. The band at 222 nm which is charac-
teristic of the right handed α-helix was present in spectra of nei-
ther human nor pig enzymes. The few spectra recorded at pH 4
and the absorption measurements at pH 13 indicate that the
high activity pig enzymes are more sensitive to acid denatura-
tion than the low activity enzyme, and, on the other hand, the
high activity enzyme apparently is more stable at the high pH.
The reverse seems to apply to the human enzymes (*Beychok
et al.*).

ADDENDUM

When preparation of the present manuscript was essentially ter-
minated, two studies of porcine erythrocyte carbonic anhydrases
were published. *Tanis et al.* (1970) have isolated and in part charac-
terized one low activity and one high activity enzyme form. A study
by *Ashworth et al.* (1971) was concentrated on a low activity form.
Findings in these studies and the results presented in this paper sup-
plement and confirm each other, but a comparison unveils some
interesting differences which will be discussed in some detail else-
where (*Hejgaard* 1971).

Based on a comparison of the recorded amino acid compositions
it is concluded that the same molecular form of the low activity type
enzyme was studied by all three workers. Either of the two high
activity enzyme preparations from Danish pigs showed a composition
identical with that of the high activity isoenzyme isolated by *Tanis
et al.*

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SAMMENDRAG

Isolering og karakterisering af tre carboanhydraser fra svineerythrocyter.

Tre distinkte former af det zinkholdige enzym carboanhydrase blev isoleret fra svineerytrocyter. Som fundet hos andre pattedyr

indeholder svinets erytrocyter to typer carboanhydrase isoenzymer med forskellige kemiske, fysiske og katalytiske egenskaber. Et lav-aktivt enzym med et isoelektrisk pH på 7,15 og et højaktivt enzym med et isoelektrisk pH på 5,97 og en 8 gange højere CO_2-hydratase-aktivitet isoleredes fra svin af Dansk Landrace. Fra danske sortbro-gede svin kunne yderligere isoleres en genetisk variant af det høj-aktive enzym.

Isoleringsproceduren omfattede en fældning af hæmoglobinet med chloroform-ethanol og separation af isoenzymerne ved DEAE-Sephadex chromatografi. De tre enzymer opførte sig som homogene proteiner ved isoelektrisk focusering og elektroforese ved forskellige pH-værdier. Det lavaktive enzym udgjorde en fjerdedel af det aktive protein i alle chloroform-ethanol ekstrakter. Under fraktioneringen observeredes et stort antal enzymatisk aktive „minor components", som tilsyneladende alle var genetisk koblet med en af de tre hovedkomponenter.

De tre oprensede isoenzymer blev karakteriseret med hensyn til molekylvægt, zinkindhold, aminosyresammensætning, ekstinktionskoef-ficient og enzymatisk aktivitet overfor CO_2, p-nitrophenylacetat og β-naphthylacetat. Enzymernes cirkular dichroisme blev også studeret. For de fleste af de studerede egenskabers vedkommende konstateredes tydelig lighed med isoenzymer af tilsvarende type isoleret fra andre pattedyr. Det lavaktive enzym indeholdt ingen svovlholdige amino-syrer. De to højaktive mutanter udviste næsten identisk aminosyre-sammensætning. Den eneste forskel, som blev observeret, var, at en argininrest i den mest udbredte variant var udskiftet med en histidin-rest i den sjældnere variant. Bortset herfra konstateredes ingen signi-fikante forskelle i de to mutanters egenskaber.

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