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Tissue and species distribution of the secreted carbonic anhydrase isoenzyme

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The secreted carbonic anhydrases, CA VI, are high molecular mass, oligomeric enzymes originally found in the sheep parotid gland and saliva. The enzymes have been purified from the saliva or parotid glands of several different species. All the CA VI enzymes studied have an apparent subunit M_r of about 45000 as previously reported for the sheep enzyme. By Western analysis, CA VI from human, cow and dog cross-reacted with antibody raised against the purified sheep enzyme whereas that of the mouse did not. The *N*-terminal sequences of the sheep, human, cow and mouse enzymes are reported. The sheep, cow and human *N*-terminal sequences are similar to one another while the mouse sequence is substantially different. Nevertheless, the amino acids in the aromatic cluster I (Trp-5, Tyr-7, Trp-16 and Tyr/Phe-20) have all been conserved, as is the case with the cytoplasmic carbonic anhydrases. Eighteen tissues from the sheep have been examined for the presence of CA VI by Western analysis but it has been found only in the salivary glands. Northern analysis and hybridization histochemistry show that the mRNA for CA VI in sheep is expressed specifically in the acinar cells of the parotid and submandibular glands.

INTRODUCTION

Several different classes of carbonic anhydrase (carbonate hydro-lyase; EC 4.2.1.1) are known. Isoenzymes I, II and III have been characterized thoroughly and are cytoplasmic, monomeric enzymes with an M_r of about 30000. The amino acid sequences of several of these proteins from different species are known [1,2]. Membrane-bound carbonic anhydrases (CA IV) have not been as well-characterized but are known to be glycoproteins with apparent subunit M_r of 52000–68000 and are solubilized only by detergent treatment [3,4]. A carbonic anhydrase has been isolated recently from guinea pig hepatocyte mitochondria [5] and its *N*-terminal amino acid sequence determined [6]. This represents a separate isoenzyme and has been classified as CA V [2]. The salivary carbonic anhydrases, CA VI, are secreted proteins first isolated from sheep parotid glands and saliva [7,8]. The enzyme has been characterized from the sheep [9], rat [10] and human [11]. These enzymes are oligomeric [9] with an apparent subunit M_r of about 45000, and contain carbohydrate [9–11]. Recently we have determined the complete amino acid sequence of the sheep enzyme [12] and this showed that the salivary enzyme is a single polypeptide chain of 307 amino acids (compared with 259–260 for the cytoplasmic isoenzymes) and has two glycosylation sites and a single intramolecular disulphide bridge. It has about 33% sequence similarity with the CA II isoenzymes, with all residues involved in the active site conserved.

A limited number of species have been examined for the presence of this enzyme. We first reported its presence in sheep and goats [7,8] and it has subsequently been described in rat [10] and human [11] saliva. This paper reports the purification of the enzyme from five species and examines how closely they are related by antibody cross-reactivity (Western analysis), and compares the *N*-terminal amino acid sequences of four of these enzymes with that of the human CA II isoenzyme. The tissue distribution of this isoenzyme in the sheep is also examined.

EXPERIMENTAL

Materials

Parotid glands were dissected from male mongrel dogs and female mice. Saliva was collected from normal sheep and cows by cannulation of the parotid duct, and from humans by expectoration following stimulation by a 2% citric acid solution. In all cases the saliva was collected into a bottle containing sufficient aprotinin and benzamidine to give final minimum concentrations of 1 μg/ml and 1 mg/ml respectively. The bottles were kept on ice while the saliva was collected. Various tissues were collected from Merino sheep immediately following death and were frozen in liquid N_2.

Acrylamide, bisacrylamide, *NNN'N'*-tetramethylethylenediamine, SDS, affinity-purified goat anti-(rabbit IgG)–horseradish peroxidase conjugate and HRP colour developer reagent were obtained from Bio-Rad. Immobilon was from Millipore and nitrocellulose was from Schleicher and Schull. Antibodies to sheep CA VI were raised as previously described [9] and were affinity purified on CA VI–Sepharose. All sequencing reagents were from Applied Biosystems. Aprotinin and pyroglutamate aminopeptidase were from Boehringer-Mannheim and other reagents used were of analytical grade.

Enzyme purification

CA VI was purified from the parotid glands by the method previously described for the sheep enzyme [9]. CA VI was purified from saliva by a modification of this procedure. Saliva was filtered (0.45 μm filter) and con-

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centrated about 10-fold by ultrafiltration, and then loaded on to the sulphonamide–Sepharose affinity column. All subsequent steps were as described previously [9]. Purity and apparent subunit M_r were determined by polyacrylamide-gel electrophoresis in the presence of 0.1% SDS as described previously [9]. Gels were silver-stained as described by Merril *et al.* [13].

Sequence analysis

The cow, human and mouse enzymes (150–200 pmol) were methanol-precipitated at −20 °C, and then sequenced on an Applied Biosystems 470A protein sequencer using the on-line 120 A phenylthiohydantoin-amino acid analyser to identify the residues. The glass fibre disc was loaded with 3 mg of Polybrene as carrier and precycled before the sample was loaded. Each protein was sequenced at least twice. The blocked cow CA VI (0.5 mg) was digested with pyroglutamate aminopeptidase (5 μg) in 0.1 M-phosphate buffer (pH 8.0), containing 5 mM-dithiothreitol, 10 mM-EDTA and 5% (v/v) glycerol for 24 h at 20 °C under N_2 atmosphere. The deblocked protein was purified by h.p.l.c. (C_4 Delta-pak (Waters); buffer A: 0.1% trifluoroacetic acid; buffer B, 80% acetonitrile/0.1% aqueous trifluoroacetic acid; linear gradient 20% B to 80% B) before sequence analysis. All sequence runs were performed with the use of the 03 PTH program.

Immunoblot analysis

The purified enzymes from the different species were run on a polyacrylamide gel as described above and electroblotted on to sheets of Immobilon using a modification [14] of the procedure of Towbin *et al.* [15]. For the study on tissue distribution, the following tissues were used: liver, adrenal gland, kidney cortex and medulla, sublingual, submandibular and parotid salivary glands, lung, pancreas, skeletal muscle, small intestine, abomasum, cortex of the brain, pituitary, choroid plexus, ovary and heart (ventricle and atrium). The tissues (~ 1 g) were homogenized in 10 ml of 0.1 M-phosphate buffer (pH 7.4) containing 5 mM-EDTA, 5 mM-benzamidine and 5 μg of aprotinin/ml, and centrifuged at 100000 *g* for 60 min. The supernatants were diluted with homogenization buffer to give an absorbance at 280 nm of about 3.0. Samples were boiled (2 min) in 1% SDS and 2% 2-mercaptoethanol, and 10 μl aliquots (2 μl for parotid homogenate) were loaded on to 10.5% polyacrylamide gels containing 0.1% SDS. After electrophoresis, the samples were electroblotted on to Immobilon as described above. Filters were allowed to react with affinity-purified rabbit anti-(sheep CA VI) antibody. The antigen–antibody complexes were visualized using the Bio-Rad horseradish peroxidase method.

Northern analysis and hybridization histochemistry

An oligodeoxynucleotide probe (56 bases long), corresponding to the first 19 amino acids of sheep CA VI [12], was synthesized by the solid-phase phosphoramidite method [16] using the most common codon choice for mammalian proteins. This was determined using a codon usage program with the nucleic acid sequence data base (NBRF) and the sequence is shown in Fig. 1. The probe was 5′-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase [17]. RNA was isolated by homogenization of tissues in 5 M-guanidinium thiocyanate followed by centrifugation through a caesium chloride cushion [18]. Polyadenylated RNA was purified by subjecting total RNA to oligo(dT)–cellulose chromatography [19]. Polyadenylated RNAs were electrophoresed through 1.2% agarose gels containing 2.2 M-formaldehyde, transferred to nitrocellulose and hybridized with the ^{32}P-labelled oligonucleotide [20]. After hybridization, the filter was washed with 2 × SSC (0.3 M-NaCl/0.03 M-sodium citrate) containing 0.1% SDS at 37 °C and subjected to autoradiography for 7 days at −70 °C with an intensifying screen. Hybridization histochemistry was carried out on sections of sheep tissue as described by Penschow *et al.* [21].

RESULTS

Purification of carbonic anhydrases

Using procedures developed to purify sheep CA VI, the enzyme was purified from cow and human saliva and from the parotid glands of the mouse and dog. For sheep CA VI, the protein was purified 7.2-fold from saliva in a 25% yield, and this gave 6.5 mg of pure protein from 2.5 litres of saliva. All of these proteins were purified to homogeneity as demonstrated by polyacrylamide-gel electrophoresis (Fig. 2). The sheep, cow, human and dog CA VI preparations all had an apparent subunit M_r of 45000, while that of mouse was about 42000.

Western analysis

The immunopurified antibody raised against sheep CA VI cross-reacted strongly with cow, human and dog CA VI but not with mouse CA VI.

N-Terminal sequence

Human and cow CA VI appeared to be blocked when subjected to *N*-terminal sequence analysis. The human CA VI sequence shown in Fig. 3 is derived from the nucleotide sequence of a cDNA clone coding for human CA VI (P. Aldred & R. T. Fernley, unpublished work). At the position equivalent to the *N*-terminus in the sheep enzyme there is a glutamine residue in the human sequence and this appears to be in the pyrrolidone carboxyl form in the native protein. The cow CA VI was digested with the enzyme pyroglutamate aminopeptidase to remove pyroglutamate residues, then sequenced. A clear amino acid sequence was then obtained and it is assumed that this carbonic anhydrase has a pyroglutamate residue at the *N*-terminus. The mouse CA VI has a free *N*-terminus. The four CA VI sequences are

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Protein	Gly	His	Gly	Val	Glu	Trp	Thr	Tyr	Ser	Glu	Gly	Met	Leu	Asp	Glu	Ala	His	Trp	Pro
mRNA	5'- GGN	CA$^{U}_{C}$	GGN	GUN	GA$^{A}_{G}$	UGG	ACN	UA$^{U}_{C}$	AC$^{N}_{UG}$	GA$^{A}_{G}$	GGN	AUG	C$^{}_{UN}$	GA$^{U}_{C}$	GA$^{A}_{G}$	GCN	CA$^{U}_{C}$	UGG	CCN -3'
Probe	3'- CCG	GTG	CCG	CAC	CTC	ACC	TGG	ATG	AGG	CTC	CCG	TAC	GAC	CTG	CTC	CGG	GTG	ACC	GG -5'

Fig. 1. *N*-Terminal sequence of sheep CA VI, the possible codons for this sequence and the sequence of the complementary oligonucleotide synthesized for use as a probe for sheep CA VI mRNA

shown in Fig. 3 and are compared to the sequence of human CA II.

Tissue distribution

Of the eighteen tissues of the sheep which were examined for the presence of CA VI by immunoblot analysis, only the parotid and submandibular salivary glands showed detectable amounts of CA VI (Fig. 4). The parotid had a higher content than the submandibular gland, while the sublingual gland had no detectable CA VI. The skeletal muscle showed a positive band with an M_r of about 30000, but no bands with an M_r of 45000.

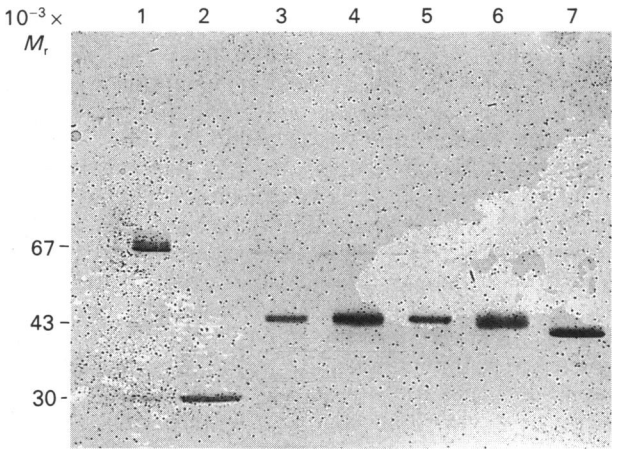


Fig. 2. Polyacrylamide-gel electrophoresis of CA VI from different species

Purified enzymes (0.5 μg) were run on a 10.5% polyacrylamide gel containing 0.1% SDS with M_r standards, fixed in 20% trichloroacetic acid and silver stained. Lane 1, M_r standards; lane 2, sheep CA II; lane 3, sheep CA VI; lane 4, human CA VI; lane 5, dog CA VI; lane 6, cow CA VI; lane 7, mouse CA VI.

Northern analysis and hybridization histochemistry

The oligonucleotide probe designed from the known protein sequence of sheep CA VI (Fig. 1) hybridized to mRNA (about 1.45 kb in length) in parotid and submandibular gland but not to mRNA from liver (Fig. 5). It also cross-hybridized to human CA VI mRNA in a human salivary gland mRNA preparation (lane 4). This probe hybridized strongly to sections of sheep parotid using the technique of hybridization histochemistry (Fig. 6*a*). This labelling was greatly reduced if unlabelled nucleotide was included in the hybridization reaction (Figs. 6*b* and 6*c*), or if the tissue section was

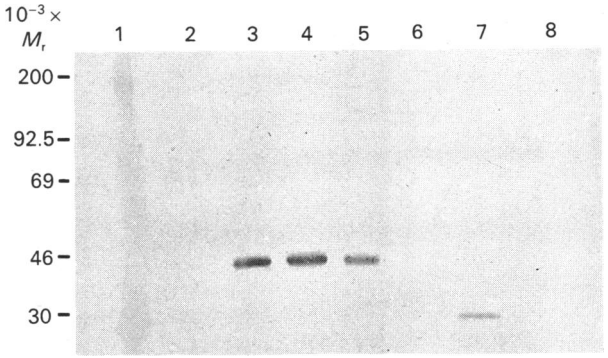


Fig. 4. Immunoblot analysis of CA VI from different tissues of the sheep

The samples were run as described for Fig. 2 and blotted onto Immobilon. The Immobilon membrane was probed with affinity-purified sheep CA VI antibody and the antigen–antibody complex visualized as described in the Experimental section. Lane 1, 'rainbow' M_r markers; lane 2, 1 μg of sheep CA II; lane 3, 0.25 μg of sheep CA VI; lane 4, 2 μl of parotid extract; lane 5, 10 μl of submandibular gland extract; lane 6, 10 μl of sublingual gland extract; lane 7, 10 μl of skeletal muscle extract; lane 8, 10 μl of kidney extract. The other tissue extracts, which were negative, are not shown.

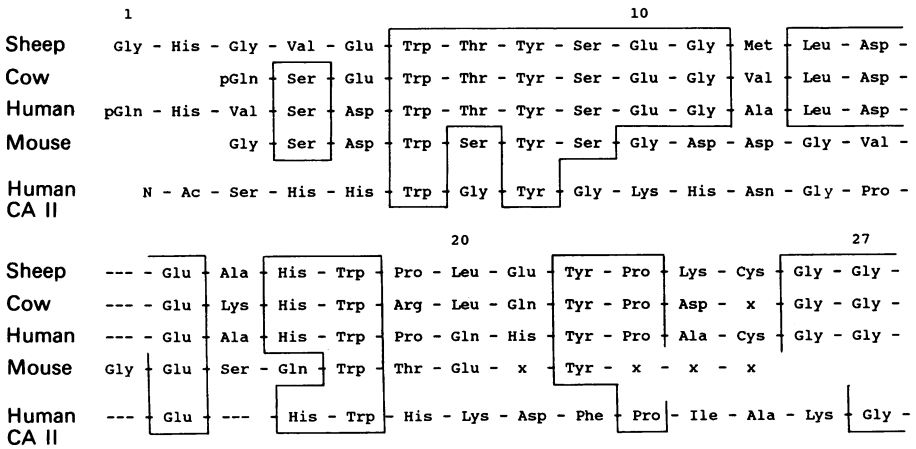


Fig. 3. Comparison of the *N*-terminal sequences of salivary carbonic anhydrases from four species with the human CA II sequence

The sequence of sheep CA VI has been reported previously [12]; the human CA VI was *N*-terminally blocked and this sequence was derived from the cDNA nucleotide sequence (P. Aldred & R. T. Fernley, unpublished work). The mouse and cow sequences were determined by automated Edman analysis, with that of the cow following enzymic removal of the *N*-terminal pyroglutamate residue, and the human CA II sequence is from ref. [22]. Gaps have been introduced into the sequences to maximize alignment.

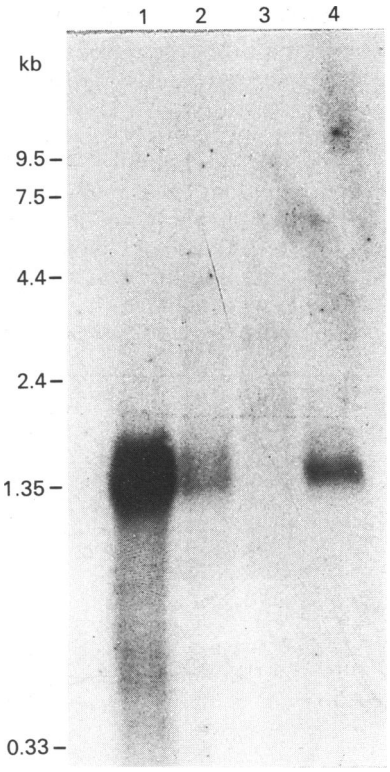


Fig. 5. Northern analysis of salivary gland carbonic anhydrase

Approximately 1 μg of each of the following mRNA preparations was subjected to Northern analysis using the ^{32}P-labelled oligonucleotide probe: lane 1, sheep parotid gland mRNA; lane 2, sheep submandibular gland mRNA; lane 3, sheep liver mRNA; lane 4, human submandibular gland mRNA. Sizes of markers are shown.

pretreated with RNAase (Fig. 6*d*). Fig. 7(*a*) shows a cross-section of sheep parotid gland with the duct cells indicated by the arrows. The rest of the cells in the section are mainly acinar cells. Fig. 7(*b*) shows an adjacent section hybridized to the ^{32}P-labelled oligonucleotide and viewed under dark-field illumination. The acinar cells and not the duct cells are labelled by this method.

DISCUSSION

The salivary or secreted carbonic anhydrases constitute a new class of carbonic anhydrase, designated CA VI. We found the enzyme originally in sheep saliva and parotid glands, and purified and characterized the enzyme from the latter source [8,9]. What appeared to be the same isoenzyme was purified also from rat [10] and human [11] saliva. Recently we reported the complete amino acid sequence of sheep CA VI [12]. We have shown here that the enzyme occurs in the saliva and salivary glands of a number of mammalian species, and by Western analysis these enzymes are, with the exception of the mouse CA VI, closely related. A comparison of *N*-terminal sequences of these proteins confirms their close relationship. Although the mouse sequence differs somewhat from the other CA VI sequences, it still has a higher degree of identity with them than with, for example, CA II. The amino acids in the aromatic cluster I of carbonic anhydrase, Trp-5, Tyr-7, Trp-16 and Tyr/Phe-20 (using the numbering system for CA I) have been conserved in all these carbonic anhydrases [23]. The CA VI isoenzyme differs in a number of properties from other CA isoenzymes. It has a higher M_r than the cytoplasmic isoenzymes (45000 versus 30000), it is glycosylated [9–11] and has an intramolecular disulphide bond [12]. There is only 33% sequence identity between sheep CA VI and sheep CA II [12], compared to about 60% identity between the different cytoplasmic isoenzymes. This implies that the CA VI gene diverged from the ancestral CA gene at a much earlier time than the divergence of the cytoplasmic isoenzymes (I, II and III), or has changed at a faster rate.

The secreted carbonic anhydrases do have some properties in common with the membrane-bound isoenzymes (CA IV). These also have a higher M_r than the cytoplasmic isoenzymes (52000–65000) and are glycoproteins [3,4]. The bovine lung enzyme also appears to have a disulphide bond [4]. However, these enzymes are membrane-bound and solubilized only by detergent treatment, unlike the freely soluble salivary enzymes.

Of the eighteen sheep tissues examined for the presence of CA VI, only the parotid and submandibular glands were positive with about ten times as much enzyme in the parotid as the submandibular gland. As can be seen from the purification procedure, the enzyme is a major constituent of sheep parotid saliva (about 14% of the protein). The oligonucleotide probe designed from the known sequence of sheep CA VI hybridizes to mRNA in parotid and submandibular gland, and this was used to localize CA VI synthesis using hybridization histochemistry. Intense labelling occurred over the acinar cells of the parotid with little or no labelling over the duct cells. The parotid gland is a serous salivary gland while the submandibular is a mixed (serous and mucous) gland. The sublingual is a mucous gland. From these studies it appears that CA VI is synthesized by only the serous acinar cells of salivary glands and from here is secreted into saliva.

Skeletal muscle showed a band with an M_r of 30000 by Western analysis, and this appears to be CA III which is an abundant protein of muscle tissue [24]. This is a surprising finding given that CA III does not appear to have a greater overall sequence identity with CA VI than does CA II, although the epitope(s) recognized by this antibody may be similar in CA III and CA VI.

Several studies on the immunohistochemical and histochemical localization of carbonic anhydrase in salivary glands have been carried out, but the identity of the secreted isoenzyme was not known and this led to confusion in interpreting results. Different groups have also reported different distributions of CA I and CA II in the salivary glands. Hennigar *et al.* [25] found most staining in the acinar cells of the parotid and submandibular glands of the mouse and rat using CA I and CA II antibodies. Noda *et al.* [26] found most immunoreactivity in the striated duct and granular convoluted tubule cells in mouse submandibular glands with CA I and CA II antibodies. Ikejima & Ito [27] raised antibodies against a carbonic anhydrase (isoenzyme unspecified) purified from mouse salivary glands for their immunohistochemical study. They found immunohistochemical staining in the secretory ducts and in the acinar cells particularly near the secretory granule membrane with no staining in the cytoplasm. In contrast, their histo-

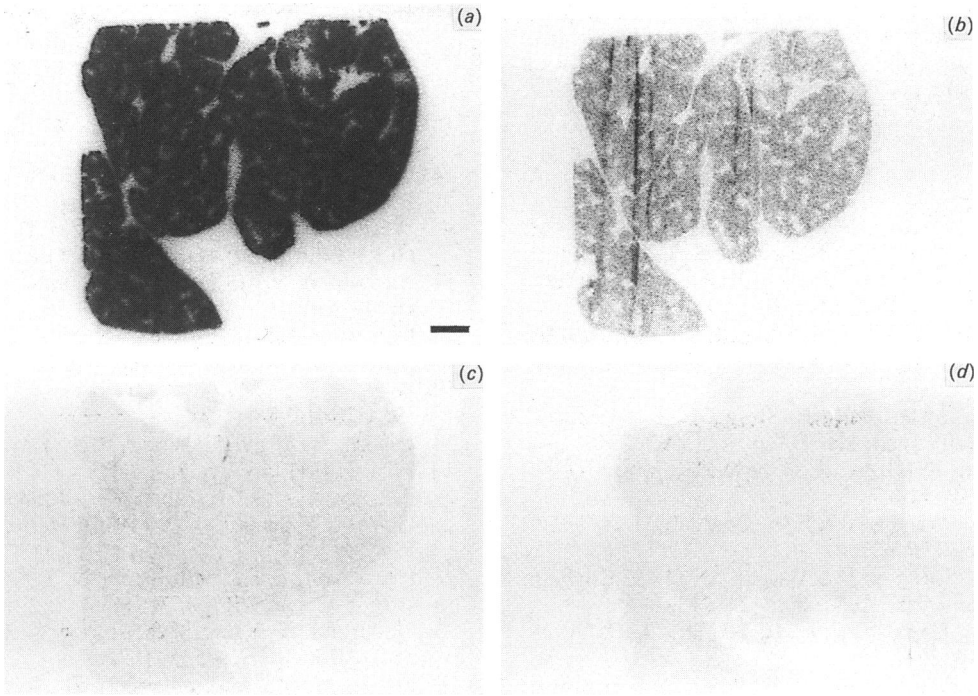
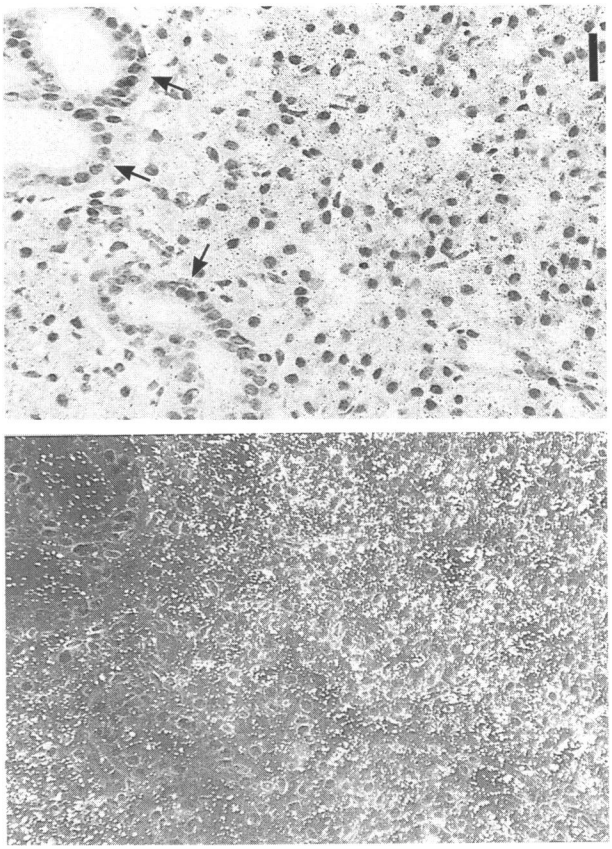


Fig. 6. Autoradiographs localizing mRNA for carbonic anhydrase VI in sheep parotid gland

X-ray film autoradiographs of 6 μm-thick frozen sections from sheep parotid gland after hybridization with (*a*), 10 ng of ^{32}P-labelled 56-mer oligodeoxyribonucleotide probe, specific for ovine carbonic anhydrase VI (bar = 1 mm); (*b*) and (*c*), 10 ng of the ^{32}P-labelled probe diluted with 100 ng (*b*) and 1000 ng (*c*) of identical unlabelled probe; and (*d*) 10 ng of radioactively labelled probe as in (*a*) after the sections were treated for 30 min at 37 °C with 20 μg of ribonuclease A/ml (Boehringer-Mannheim) in 0.01 M-Tris buffer, pH 7.5.



chemical staining for carbonic anhydrase activity was mainly in the cytoplasm of the acinar cells as well as in the secretory ducts. It is possible that their antibody was raised against CA VI and this has labelled the secretory granules and secreted enzyme in the ducts. Their histochemical staining appears to have revealed CA II (or CA I) in the cytoplasm of the acinar cells and CA VI in the secretory ducts but not CA VI packaged in the secretory granules of the acinar cells. Our results confirm that CA VI is synthesized in the acinar cells of the parotid from where it is secreted into the saliva.

Isolation and characterization of the gene coding for a secreted carbonic anhydrase would yield valuable new information on the structure of this new class of carbonic anhydrase and its relationship to other members of the carbonic anhydrase gene family.

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Fig. 7. Liquid emulsion autoradiographs of sheep parotid tissue with sheep carbonic anhydrase VI probe

mRNA for ovine CA VI demonstrated in serous acinar cells of the sheep parotid gland using the identical ^{32}P-labelled probe shown in Fig. 6. Liquid-emulsion autoradiographs photographed by light-field (*a*) and dark-field (*b*) microscopy. Arrows indicate unlabelled ducts. Stain: haematoxylin and eosin. Bar = 25 μm.

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