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Carbonic Anhydrase Isoenzymes in the Erythrocytes and Uterus of the Rabbit

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1. Two forms of the zinc-containing enzyme carbonic anhydrase (EC 4.2.1.1) were isolated from rabbit erythrocytes and two forms from rabbit uterine tissue (endometrium) in the progestational stage of pregnancy (days 6–8 of gestation). Separation of the isoenzymes was achieved by ion-exchange chromatography, preparative polyacrylamide-gel electrophoresis and isoelectric focusing. A comparison was made of the general properties and kinetic behaviour of the purified isoenzymes. 2. Although indistinguishable in terms of molecular weight and zinc content the isoenzymes were very different as catalysts of the hydration of carbon dioxide. The two erythrocyte isoenzymes, found in almost equal amounts, differed more than 100-fold in specific activity. Of the two isoenzymes prepared from either endometrial or entire uterine homogenates one was kinetically indistinguishable from the erythrocyte high-activity form, whereas the other, also possessing high activity, was found only in the endometrial or uterine tissue. Present evidence suggests that the former isoenzyme originated from residual blood contaminating the tissue homogenates, and that a marked rise in the content of the latter isoenzyme accounts for the increase in rabbit endometrial carbonic anhydrase activity that previously has been observed in early pregnancy. 3. Minor forms of the erythrocyte isoenzymes, having a characteristic quantitative and electrophoretic relationship to one another, were occasionally produced during purification. 4. The actions were investigated of the inhibitors acetazolamide (5-acetamido-3,4-diazole-1-thia-2-sulphonamide), 1,1-dimethylaminonaphthalene-5-sulphonamide and ethoxyzolamide (6-ethoxybenzothiazole-2-sulphonamide) on the hydration of carbon dioxide and the hydrolysis of *p*-nitrophenyl acetate catalysed by the isoenzymes. 5. The low-activity erythrocyte isoenzyme was superior to the high-activity form as a catalyst of β-naphthyl acetate hydrolysis.

Multiple forms of the zinc-containing enzyme carbonic anhydrase (EC 4.2.1.1) were first detected in bovine (Lindskog, 1960) and human erythrocytes (Nyman, 1961; Rickli & Edsall, 1962; Laurent, Marriq, Nahon, Charrel & Derrien, 1962). Carbonic anhydrase is an excellent catalyst of the reversible hydration of CO_2 and, to a lesser extent, of the hydration of other carbonyl compounds (Pocker & Meany, 1965; Pocker & Dickerson, 1968), the hydrolysis of several types of ester (Pocker & Stone, 1965, 1968) and the hydrolysis of a sultone (Kaiser & Lo, 1969).

Although carbonic anhydrase activity has been detected in the endometrium of the non-pregnant (oestrous) rabbit uterus its activity reaches a peak 6–8 days after mating in the so-called progestational phase of pregnancy (Lutwak-Mann, 1954) or after an ovulating dose of gonadotrophin (Lutwak-Mann, 1955). Progesterone is the principal ovarian hormone responsible but various luteoids with progesterone-like activity are equally capable of exerting this stimulation (Lutwak-Mann & Adams, 1957). A precise method of assay of the enzyme has recently been used to investigate the relationship between endometrial carbonic anhydrase and the content of zinc in this tissue (Lutwak-Mann & McIntosh, 1969).

The present paper describes the purification, characterization and comparison of isoenzymes of carbonic anhydrase from the erythrocytes and uterus of the rabbit. The evidence accumulated suggests that a specific endometrial carbonic anhydrase isoenzyme is present in the progestational, but not the non-pregnant, rabbit endometrium.

MATERIALS AND METHODS

Reagents. Tris (reagent grade), *p*-nitrophenyl acetate, β-naphthyl acetate, Fast Blue RR and soya-bean trypsin inhibitor were obtained in a satisfactory state of purity from Sigma (London) Chemical Co., London S.W.6, U.K. Diethylmalonic acid was prepared by the hydrolysis of diethyl diethylmalonate which, together with the reagents used in the polyacrylamide-gel electrophoresis, were supplied by Kodak Ltd., Kirkby, Lancs., U.K. The method of Weber (1952) was used to prepare DNSA* from the sulphonyl chloride (BDH Chemicals Ltd., Poole, Dorset, U.K.). DEAE-Sephadex A-50, Sephadex G-100 and G-200 and Blue Dextran were all obtained from Pharmacia (G.B.) Ltd., London W.13, U.K. Coomassie Blue (Brilliant Blue R250) was supplied by George T. Gurr Ltd., London S.W.6, U.K. Diamox, the sodium salt of acetazolamide (5-acetamido-3,4-diazole-1-thia-2-sulphonamide), assumed to have an equivalent weight of 270 (Whitney, Fölsch, Nyman & Malmström, 1967) was the product of the Cyanamid Co., Pearl River, N.Y., U.S.A., and ethoxyzolamide (6-ethoxybenzothiazole-2-sulphonamide) was a gift from the Upjohn Co., Kalamazoo, Mich., U.S.A.; both inhibitors were used without further purification. Myoglobin, ovalbumin and bovine carbonic anhydrase isoenzyme B were generously given by Seravac Laboratories Ltd., Maidenhead, Berks., U.K., Dr E. F. Hartree and Dr J. C. Kernohan respectively. Human erythrocyte carbonic anhydrase isoenzymes B and C were prepared by the method of Armstrong, Myers, Verpoorte & Edsall (1966). Most other chemicals were of analytical grade and the water used was deionized or glass-distilled.

Assay of hydratase activity towards CO_2. Precise measurement of the initial hydration rate was carried out by titration at constant pH (McIntosh, 1968). The assay mixture (18.0ml), maintained at 0°C, contained 5mM-sodium phosphate, 45mM-NaCl, 0.01% peptone and enzyme, at pH7.00. Reaction was initiated by the addition of CO_2, as an aqueous solution of the gas saturated at 0°C, and the hydration rate was recorded. Allowance was made for the rate of hydrolysis in the absence of enzyme. In the experiments with inhibitors, the enzyme and inhibitor were mixed 5min before the addition of substrate. A unit of carbonic anhydrase activity is defined as the amount that catalyses the hydration of 1μmol of CO_2/min under the assay conditions stated above, with a concentration of 1mM-CO_2. Because of the limited solubility of CO_2 in water it was impossible to use a concentration sufficient to saturate the enzyme.

Enzyme activity was determined in the effluents from column chromatography, preparative electrophoresis and isoelectric focusing by the method of Philpot & Philpot (1936). This assay gives only an approximate measure of CO_2 hydratase activity and is unsuitable for the determination of kinetic constants.

Spectrophotometric assays of esterase activity with the substrates p-*nitrophenyl acetate and* β-*naphthyl acetate.* Details of both methods are given by McIntosh (1969).

DEAE-Sephadex ion-exchange chromatography. DEAE-Sephadex A-50 was swollen in water and washed with dilute acid and alkali, according to the manufacturer's instructions, before equilibration with the desired buffer at 4°C. Three buffers were used: 0.1M-tris–24mM-HCl (pH9.3 at 4°C), and the same buffer diluted twofold and fourfold with water. These buffers were named I, II and III respectively. The prepared gel was packed to form columns 35cm long with a diameter appropriate to the particular experiment. The enzyme was eluted with the buffer with which the particular column had been equilibrated, by the method of Armstrong *et al.* (1966). All chromatography was done at 4°C. Pooled fractions of effluent were concentrated by freeze-drying after exhaustive dialysis against deionized water, or by evaporation from dialysis sacs at 4°C.

Isoelectric focusing. Details are given by McIntosh (1969). The 110ml-capacity apparatus and the carrier ampholyte mixtures used were manufactured by LKB-Produkter AB, Stockholm, Sweden.

Preparative polyacrylamide-gel electrophoresis. The apparatus supplied by Shandon Scientific Co. Ltd., London N.W.10, U.K. was used. Generally, the manufacturer's instructions were followed. The sample, containing sucrose and Bromophenol Blue (to indicate the emergence from the column of the 'front'), was layered directly on to the small-pore (7.5% acrylamide) gel prepared as described by Davis (1964). Electrophoresis was carried out for 12–36h in the discontinuous buffer system with a constant current of 30mA. The apparatus was cooled to 4°C by circulating water. The rate of flow of the eluting buffer was about 15ml/h and 2ml fractions were collected.

Analytical polyacrylamide-gel electrophoresis. For electrophoresis at pH9.5 the method of Davis (1964) was used except that the sample and spacer gels were omitted; other details are given by McIntosh (1969). Electrophoresis at pH5 was done by the method of Reisfeld, Lewis & Williams (1962) with 7.5% polyacrylamide and again the spacer and sample gels were omitted. Protein was stained with Coomassie Blue and esterase activity towards β-naphthyl acetate at pH7.0 was detected by coupling Fast Blue RR with the released β-naphthol (Tashian & Shaw, 1962). Acetazolamide was added to control gels to inhibit carbonic anhydrase activity.

Isoelectric focusing in polyacrylamide gel. The method of Barrett (1970) was followed except that destaining of the gels was done with two changes of the solution used for staining (the Coomassie Blue being omitted) and final clarification of the gels was achieved by using 7% (v/v) acetic acid. The latter was also suitable for prolonged storage of the gels.

Determination of molecular weight by gel filtration. The method of Andrews (1964, 1965) was used. A column (50cm × 1.5cm) of Sephadex G-100 was used and details of the standards used for calibration are given by McIntosh (1969). Filtration was done at 4°C with buffer III containing 50mM-NaCl, and a Blue Dextran high-molecular-weight reference was included in every experiment.

Determination of zinc. Effluents from column chromatography were analysed directly to find the elution pattern of zinc by using a Pye–Unicam SP.90 atomic-absorption spectrophotometer. The zinc contents of samples of carbonic anhydrase dissolved in deionized water were indistinguishable from those of samples that had been digested with HNO_3 before analysis. Therefore, in these experiments, zinc determinations were done on undigested

* Abbreviation: DNSA, 1,1-dimethylaminonaphthalene-5-sulphonamide.

samples of purified enzyme. All glassware was soaked in 6M-HCl for 24h and rinsed well with deionized water before use.

Fluorescence measurements. It has been reported that the weak yellow fluorescence of DNSA becomes blue and extremely intense when the dye is conjugated with carbonic anhydrase (Chen & Kernohan, 1967). A Farrand model A filter fluorimeter (Farrand Optical Co. Inc., New York, N.Y., U.S.A.) was used to measure the similar fluorescence displayed by DNSA in combination with the rabbit carbonic anhydrase isoenzymes. Greatly increased sensitivity was achieved by blowing a hole in the glass envelope of the mercury lamp (General Electric, type H85 A3), thus increasing the output of u.v. radiation. The excitation filter was Wood's glass and the fluorescence filter was a combination of two filters of unknown types having a transmittance of 50% at 450nm decreasing to 1% at 425 and 520nm. Titration of DNSA–carbonic anhydrase in 10mM-sodium phosphate buffer, pH7.0, with 0.1mM-ethoxyzolamide was done with a 10μl Hamilton micro-syringe. The Spectrosil cuvette contained 1ml of solution.

Determination of enzyme concentration. The molar concentrations of the carbonic anhydrase isoenzymes in stock solutions were determined by titrating with ethoxyzolamide both the *p*-nitrophenyl acetate hydrolase activity and the fluorescent DNSA–carbonic anhydrase conjugates.

Estimation of residual blood content of tissue extracts. The total haematin contents of the extracts made either from endometrium or uterus described below were compared with that of haemolysates after conversion into pyridine haemochromogen (Elliott & Keilin, 1934). This method determines haem-containing compounds other than haemoglobin and so may have overestimated the haemoglobin content of the tissue extracts. The samples were treated with NaOH to give a concentration of 0.1M and after being stood for 1h sufficient pyridine was added to make a 20% (v/v) solution. The extracts were then reduced by the addition of a little $Na_2S_2O_4$. Because the extracts of endometrium and uterus were turbid it was convenient to determine the pyridine haemochromogen with a micro-spectroscope with which the intensities of the absorption bands were matched by eye. This instrument has the advantage of being unaffected by turbidity in the sample; a full description is given by Hartree (1955).

RESULTS

Preparation of the isoenzymes

All purification procedures were carried out at 4°C.

Erythrocyte enzymes. Blood was collected from cross-bred female rabbits into an excess of chilled glucose–citrate anticoagulant (Loutit & Mollison, 1943) and the erythrocytes were washed four times with cold 0.9% sodium chloride by centrifugation. After haemolysis of the erythrocytes by addition of 1.1vol. of water, carbonic anhydrase was separated from haemoglobin on columns of DEAE-Sephadex (Armstrong *et al.* 1966). In one experiment, for purposes of comparison, the haemoglobin

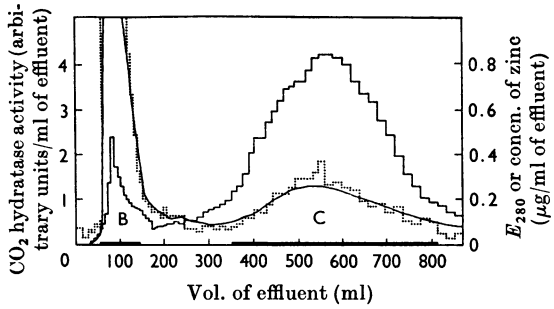

Fig. 1. Purification of erythrocyte carbonic anhydrase and resolution into isoenzymes by column chromatography on DEAE-Sephadex. The starting material was a crude extract of the enzyme after removal of haemoglobin from the haemolysate by adsorption on DEAE-Sephadex. The buffer used for equilibrating the column and eluting the isoenzymes was 50mM-tris–12mM-HCl, pH9.3 at 4°C. —— (continuous), E_{280}; —— (stepped), CO_2 hydratase activity (Philpot assay); ····, concn. of zinc. The solid bars near the horizontal axis show which fractions were combined for further purification.

was denatured and precipitated by treatment with chloroform and ethanol (Tsuchihashi, 1923). In the first, preferred, procedure the haemolysate was dialysed against buffer II and applied to a column of DEAE-Sephadex equilibrated with the same buffer. Best results were obtained if no more than half the length of the column was saturated with haemoglobin. On elution with the same buffer the major part (92%) of the enzyme, as judged by recovery of CO_2 hydratase activity, was carried from the column under these conditions. More (5%) activity was released if elution was continued with buffer I, but this brought a large part of the haemoglobin from the column.

Separation of the carbonic anhydrase isoenzymes was achieved by repeating the chromatography under identical conditions, when the component having low CO_2 hydratase activity was eluted near the void volume and the high-activity component was considerably retarded (Fig. 1). The low- and high-activity isoenzymes are referred to as Eryth. B and Eryth. C respectively to conform with the usual nomenclature (Furth, 1968). Polyacrylamide-gel electrophoresis of the pooled peaks of low and high activity revealed them to contain minor constituents. Fig. 2 shows the results of electrophoresis done at successive stages of purification and with staining for protein and β-naphthyl acetate esterase activity in the presence and absence of acetazolamide. Eryth. C was inferior to the low-activity form as a catalyst of β-naphthyl acetate hydrolysis and there was an esterase not inhibited by acetazolamide.

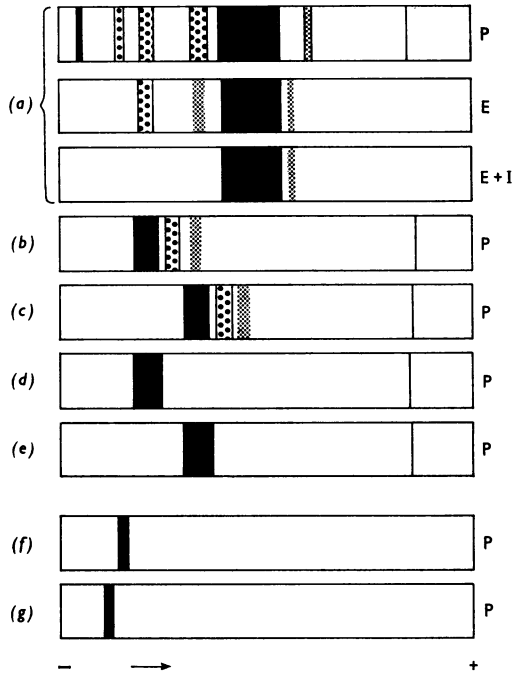

Fig. 2. Polyacrylamide-gel electrophoretograms obtained at pH 9.5 at several stages in the isolation of carbonic anhydrase isoenzymes from erythrocytes. The direction of migration of protein is indicated by the arrow. P, protein stained with Coomassie Blue; E, esterase activity towards β-naphthyl acetate at pH 7; E+I, esterase activity in the presence of acetazolamide. (*a*) Whole haemolysate of washed erythrocytes (the heavy band is haemoglobin); (*b*) preparation Eryth. B after chromatography on DEAE-Sephadex; (*c*) preparation Eryth. C after chromatography on DEAE-Sephadex; (*d*) preparation Eryth. B after preparative electrophoresis; (*e*) preparation Eryth. C after preparative electrophoresis; (*f*) and (*g*) as in (*d*) and (*e*) respectively but electrophoresis at pH 5.

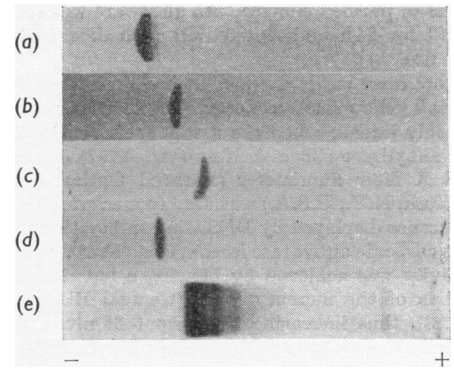

Fig. 3. Analytical isoelectric focusing of the final preparations of the isoenzymes and polacrylamide-gel electrophoresis of a special sample of preparation Eryth. C showing the 'generated' components (see the text for details). Protein was stained with Coomassie Blue. The range of the carrier ampholytes in the isoelectric-focusing experiments (*a–d*) was pH 3–10. The pH in the electrophoresis experiment (*e*) was 9.5. (*a*) Preparation Eryth. B; (*b*) preparation Eryth. C; (*c*) preparation Uterus C1; (*d*) preparation Uterus C2; (*e*) special sample of preparation Eryth. C.

Further purification was achieved by isoelectric focusing or preparative polyacrylamide-gel electrophoresis. Both methods eliminated minor contaminants; pooled preparations of Eryth. B and Eryth. C that had been prepared in either way were essentially homogeneous when subjected to electrophoresis at pH 9.5 and 5 (Fig. 2), and isoelectric focusing in polyacrylamide gel (Fig. 3).

A few samples of both isoenzymes that had passed these tests of homogeneity were subsequently revealed by the same methods to contain several additional components. On electrophoresis the separation of all of these was uniform and the intensity of protein staining formed a graduated series (Fig. 3*e*). Each band was active as a β-naphthyl acetate hydrolase and it was clear that the Eryth. B and Eryth. C components were each accompanied by minor forms with high and low activities respectively. The minor components, which could conveniently be isolated by means of preparative polyacrylamide-gel electrophoresis (Fig. 4), were presumably the result of some treatment of the protein, and, although no systematic investigation was made, it appears that freeze-drying in the presence of high salt concentrations may have been responsible, particularly with preparation Eryth. C. This study was not continued and it is not certain if the minor forms occur in haemolysates.

In the one experiment in which an alternative method was tried for separating the enzyme from haemoglobin, the haemolysate was treated with chloroform–ethanol as described by Armstrong *et al.* (1966) and the enzyme-containing extract was dialysed against water and concentrated. After dialysis of the residue against buffer II it was applied to a column of DEAE-Sephadex and the carbonic anhydrase isoenzymes were eluted in the usual way. The elution pattern was similar to the one described above, the only difference being the appearance of a small high-activity component emerging from the column between isoenzymes B and C. This minor form was not examined. The B and C components were purified and a comparison was made of the isoenzymes prepared by the two methods.

Endometrial or uterine isoenzymes. Initially carbonic anhydrase was extracted and purified on a

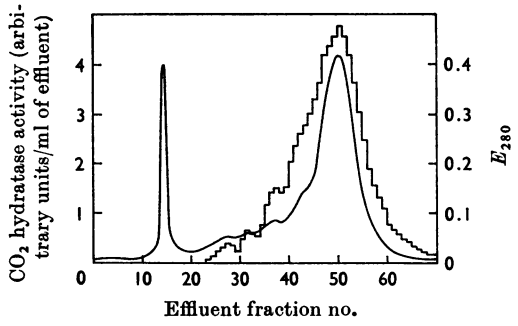

Fig. 4. Separation of isoenzyme C from minor components generated by freeze-drying in the presence of tris–HCl buffer by preparative polyacrylamide-gel electrophoresis. The rate of flow of the eluting buffer was 12ml/h, the fraction size was 2ml and the current was 30mA at 400V. —— (continuous), E_{280}; —— (stepped), CO_2 hydratase activity (Philpot assay). The sharp peak marks the emergence from the gel of Bromophenol Blue added to the sample to indicate the progress of electrophoresis.

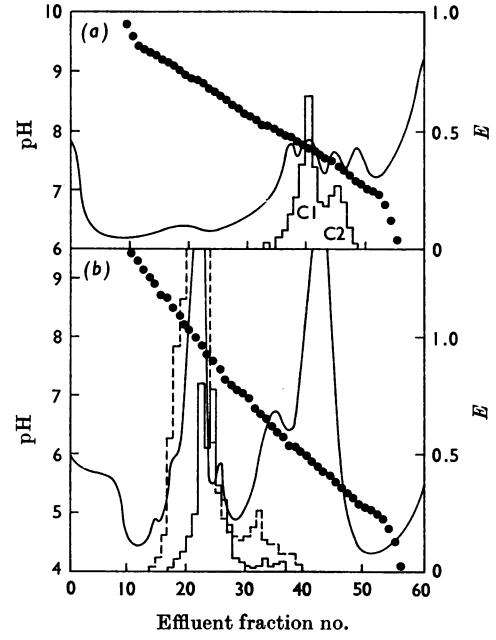

Fig. 5. Results of isoelectric focusing of (*a*) the endometrial extract of carbonic anhydrase previously subjected to DEAE-Sephadex chromatography and gel filtration, and of (*b*) the enzyme-containing $(NH_4)_2SO_4$ precipitate from the uterine extract. The fraction volume was 2ml. ●, pH; —— (continuous), E_{280}; —— (stepped), CO_2 hydratase activity (Philpot assay); ----, E_{412}, the absorption of the haemoprotein.

small scale by using endometrial tissue dissected from the rest of the uterine wall (*a*), but later the enzyme was purified by using whole uteri (*b*), and although the enzyme only occurs in the endometrium the results show that the latter course was satisfactory. Low-activity carbonic anhydrase isoenzymes that may have been present in the uterine extracts were not examined.

(*a*) The preliminary purification from endometrium was similar to the procedure used for the blood isoenzymes. Endometrial tissue (34g) was ground with 9vol. of buffer I by using acid-washed sand in a mortar and centrifuged. The supernatant (containing 70000 units of enzyme activity and 2.2g of protein) was freeze-dried and dialysed against buffer I before being loaded on to a column (20cm × 4cm) of DEAE-Sephadex equilibrated with buffer I. Elution with the same buffer produced a single ill-defined peak of anhydrase activity, and a small amount of haemoprotein was retained on the column. When elution was continued with M-sodium chloride in buffer I the haemoprotein and a further 7% of the enzyme activity were obtained; this material was discarded. The major fraction of the activity (51000 units containing 570mg of protein) was freeze-dried. The rather poor recovery (73%) probably results from the great impurity of the extract applied to the column and to its content of viscous mucoproteins, which makes gel filtration inefficient.

High-molecular-weight substances were removed from the freeze-dried active extract by gel filtration on a column (40cm × 2cm) of Sephadex G-200. The preparation (yield, 42000 units of carbonic anhydrase, 100mg of protein) was subjected to isoelectric focusing in a pH gradient (pH7–10) giving the pattern shown in Fig. 5(*a*). The two peaks of activity were designated C1 and C2 since both had high activities as CO_2 hydratases. The two peaks were applied separately to isoelectric-focusing gradients (pH6–8) and two distinct forms of carbonic anhydrase were obtained. These, designated Endomet. C1 and Endomet. C2, were judged by polyacrylamide-gel electrophoresis (Figs. 6*f* and 6*g*) to be sufficiently pure for comparison by electrophoresis and isoelectric focusing with the isoenzymes extracted from whole uteri.

(*b*) A larger-scale preparation of carbonic anhydrase from entire uterine horns was done as follows. The uteri were taken from animals that had been thoroughly bled and were carefully blotted free of blood. The tissue (233g) was frozen in liquid N_2 in small amounts and pulverised to a fine powder in a cooled stainless steel percussion mortar. The powder was suspended in 4vol. of buffer II and homogenized in a cooled Waring Blendor for 3min at full speed. The homogenate (containing 210000

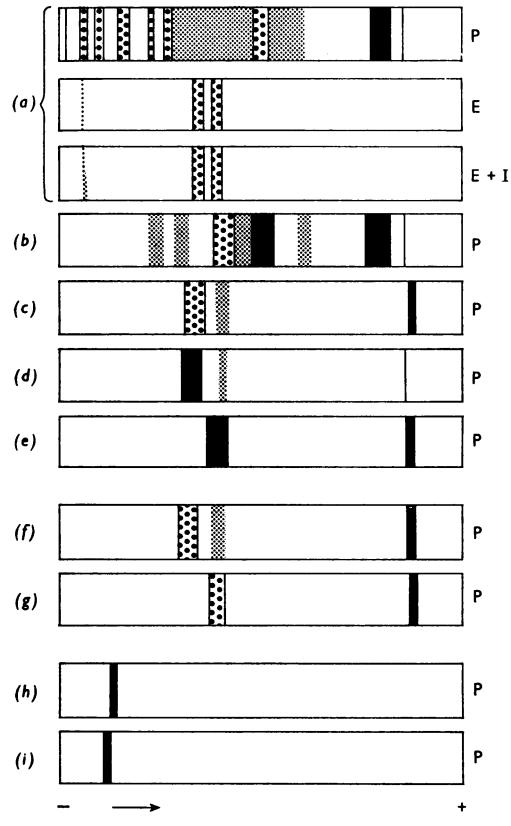

Fig. 6. Polyacrylamide-gel electrophoretograms obtained at pH 9.5 at several stages in the isolation of carbonic anhydrase isoenzymes from a soluble extract of uterine tissue. Also shown are the isoenzymes isolated from endometrial tissue homogenates. The direction of migration of protein is indicated by the arrow. P, protein stained with Coomassie Blue; E, esterase activity towards β-naphthyl acetate at pH 7; E+I, esterase activity in the presence of acetazolamide. (*a*) Whole soluble extract; (*b*) active fraction from $(NH_4)_2SO_4$ precipitation step; (*c*) preparation Uterus C1 after isoelectric focusing; (*d*) preparation Uterus C1 after preparative electrophoresis; (*e*) preparation Uterus C2 after isoelectric focusing; (*f*) preparation Endomet. C1 and (*g*) preparation Endomet. C2 after isoelectric focusing; (*h*) and (*i*), as (*d*) and (*e*) respectively but electrophoresis at pH 5.

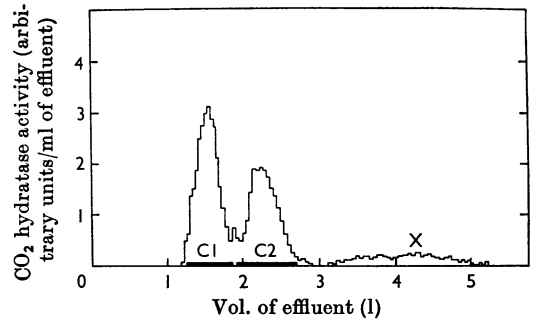

Fig. 7. Purification of carbonic anhydrase from a soluble extract of uterine tissue by column chromatography on DEAE-Sephadex. The animals were at day 6 of pregnancy. The buffer used for equilibrating the column and eluting the isoenzymes was 50 mM-tris–12 mM-HCl, pH 9.3 at 4°C. The solid bars near the horizontal axis show which fractions were combined for further purification. The two major peaks are designated C1 and C2, corresponding to the nomenclature of the isoenzymes. The minor high-activity peak X was not further purified.

units of carbonic anhydrase activity) was made to 10 vol. and stirred overnight. The supernatant obtained from centrifuging the homogenate at 12000*g* for 30 min was dialysed exhaustively against water to yield a pale pink solution, which was then clarified by centrifugation at low speed. To the 1660 ml of extract, containing 209000 units of carbonic anhydrase and 17.5 g of protein, was added 0.38 g of ammonium sulphate (containing 1 part of tris in 600)/ml, giving a 50% saturated solution, pH 8.1 at 4°C. After the suspension had been stirred for 10 min it was centrifuged, and to the supernatant was added more ammonium sulphate to give a total concentration of 0.55 g/ml based on the initial volume (73% saturated). The precipitate from this step contained 95% of the original enzyme activity together with haemoprotein, and the remaining 5% of the activity was found in the second supernatant. The active precipitate was dialysed against water to yield 109 ml of a clear, pink solution containing 200000 units of carbonic anhydrase and 2.6 g of protein.

In a pilot experiment a portion of the salt extract containing 4000 units of activity was subjected to isoelectric focusing in the range pH 3–10. The pattern shown in Fig. 5*b* suggested a similarity between the two forms of enzyme in the uterine extract with those extracted from the endometrium alone and indicated that no other major high-activity isoenzyme was present in the uterine extract.

The salt extract was chromatographed in the usual way on a column (3.5 cm diam.) of DEAE-Sephadex to give the result shown in Fig. 7. The haemoprotein was retained on the top of the column. The two large peaks of activity were named C1 and C2 in order of elution. The small fraction of the total activity eluted after peak C2 was of the high-activity type and was disregarded. The total recovery of activity was excellent (189000 units); peak C1 accounted for 58% of the activity and 6.4 mg of protein, peak C2 for 37% of the activity and

Table 1. *Summary of certain properties of the carbonic anhydrase isoenzymes*

The kinetic experiments with CO_2 as substrate were carried out at 0°C at pH 7.0 with 45mM-NaCl, 5mM-sodium phosphate and 0.01% peptone. The kinetic constants were evaluated from the results in Fig. 10; the maximum errors in the values of K_m and $V_{max.}$ are about ±10% and ±20% respectively (McIntosh, 1968). The kinetic experiments with *p*-nitrophenyl acetate and β-naphthyl acetate as substrates were done at 25°C at pH 8.0 with 10mM-diethylmalonate and either 2% (v/v) or 5% (v/v) acetone for the two substrates respectively. CEP denotes purification from a chloroform–ethanol extract.

	Erythrocyte isoenzymes				Isoenzymes extracted from the uterus	
	Eryth. B	Eryth. B (CEP)	Eryth. C	Eryth. C (CEP)	Uterus C1	Uterus C2
General properties						
Mol. wt. (±3000)	29000	29000	29000	29000	29000	29000
g-atom of Zn/mol (nearest whole number)	1	1	1	1	1	1
Iso-ionic point (from isoelectric focusing)	7.94	—	7.45	—	7.82	7.47
Kinetic properties as CO_2 hydratases						
K_m (mM)	approx. 27	>40	6.2	6.4	—	6.1
$10^{-4} \times V_{max.}/[E_0]$ (s^{-1})	approx. 0.1	—	14	6.5	—	12
Hydrolysis of 1mM-*p*-nitrophenyl acetate						
$v/[E_0]$ (min^{-1})	18	12	74	69	86	70
Hydrolysis of 1mM-β-naphthyl acetate						
$v/[E_0]$ (min^{-1})	8.0	9.0	0.51	0.55	—	—

13mg of protein and the minor peak contained 5% of the activity and 6.9mg of protein.

Peaks C1 and C2 were concentrated and applied separately to isoelectric focusing gradients (pH 6–8). After elution from the apparatus each was reconcentrated and passed through a column of Sephadex G-100 to remove the carrier ampholytes. The recovery of activity from these steps was good (about 72% overall). Polyacrylamide-gel electrophoresis revealed that preparation C1 contained a small amount of material that was probably preparation C2 (Fig. 6c); preparation C1 was further purified by preparative polyacrylamide-gel electrophoresis. The isoenzymes were found to be essentially homogeneous when subjected to electrophoresis at pH 9.5 and pH 5 and to isoelectric focusing in polyacrylamide-gel (Fig. 3) and were named Uterus C1 and Uterus C2. The mobilities of preparations Eryth. C and Uterus C2 on electrophoresis were identical under identical conditions and it was not possible to resolve a mixture of the two isoenzymes.

Properties of the isoenzymes

The properties of the isoenzymes isolated from the erythrocytes (Eryth.) and uterus (Uterus) are listed in Table 1. Neither the C1 nor the C2 isoenzyme from the endometrium could be distinguished by electrophoresis or isoelectric focusing from the C1 and C2 isoenzymes of the uterus.

Molecular weights. Gel filtration showed that all the isoenzymes had a molecular weight in the range 26000–31000, which is within the limits of experimental error. Measurements of zinc content, together with determinations of molar concentration and the assumptions of 1g-atom of zinc/mol of enzyme and a single active site lead to agreement on the molecular weight.

Measurement of enzyme concentration. The molar concentration of each enzyme in stock solutions was determined in two ways. One was to measure the variation in the initial velocity of the *p*-nitrophenyl acetate hydrolase reaction as the total concentration of enzyme was varied in an assay mixture containing a constant concentration of ethoxyzolamide, and the other was to titrate the fluorescent DNSA–enzyme complexes with ethoxyzolamide as described by Chen & Kernohan (1967). Agreement between these two methods was very good. In both instances it was necessary to assume that there was only one catalytic, or sulphonamide-binding, site on each enzyme molecule.

The *p*-nitrophenyl acetate reaction was carried out in the usual way, and it was convenient to measure enzyme concentrations in the range

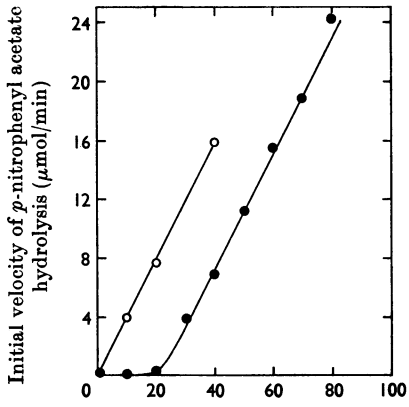

Fig. 8. Determination of the molar concentration of a solution of preparation Eryth. C by titration of a portion of the strong inhibitor ethoxyzolamide with the isoenzyme. The fraction of the isoenzyme that remained uninhibited was estimated by its catalytic effect in the *p*-nitrophenyl acetate reaction. The experiments were carried out at 25°C at pH 8.0 with 10mM-diethylmalonate, 2% (v/v) acetone and 1.0mM-*p*-nitrophenyl acetate. ○, Reaction in the absence of inhibitor; ●, reaction in the presence of 0.1 μM-ethoxyzolamide. With the assumptions described in the text the result shows that the concentration of the isoenzyme in the stock solution was 4.6 μM.

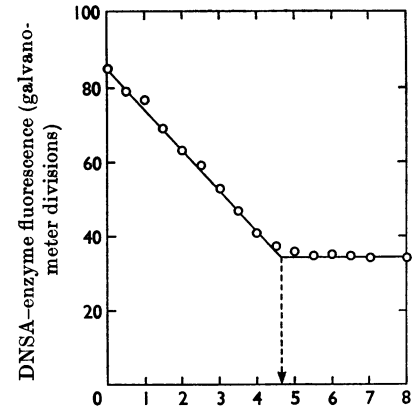

Fig. 9. Determination of the molar concentration of a solution of preparation Eryth. C by titration of the DNSA–carbonic anhydrase conjugate with ethoxyzolamide. A sample (0.10ml) of the stock solution of the isoenzyme was diluted with water and phosphate buffer to give 1.0ml of a solution containing 10mM-sodium phosphate at pH 7.0, to which was added 7 μl of 1.0mM-DNSA. With the assumptions described in the text the result shows that the concentration of the isoenzyme in the stock solution was 4.7 μM.

0.2–1 μM. The method depends on ethoxyzolamide being a tightly-bound inhibitor or, with the terminology of Webb (1963), a mutual-depletion inhibitor in zone C. An example of an inhibition experiment is shown in Fig. 8. This is a reliable way of measuring $[E_0]$ (Morrison, 1969).

The fluorescence-titration technique depends on the dissociation constant of the DNSA–enzyme complex being about 1000 times greater than that of the ethoxyzolamide–enzyme complex. Therefore when a solution of enzyme containing DNSA in molar excess was titrated with ethoxyzolamide the fluorescent DNSA–enzyme complex was quantitatively destroyed, allowing a precise determination of the amount of sulphonamide bound to the enzyme. The assumption that the active site was identical with the DNSA- and ethoxyzolamide-binding sites was borne out by the results obtained, an example of which is shown in Fig. 9. The method was checked with a sample of the bovine carbonic anhydrase B used by Chen & Kernohan (1967), generously supplied by Dr J. C. Kernohan. The displacement of DNSA from preparation Eryth. B by ethoxyzolamide appeared to be a slow reaction since about 30s elapsed before a minimum value of fluorescence was obtained after the addition of each portion of ethoxyzolamide. Chen, Schechter & Berger (1969) reported that the association of DNSA and bovine carbonic anhydrase B is a relatively slow reaction, being about 90% complete after 1s.

CO_2 hydratase activities of the isoenzymes and their inhibition by acetazolamide. The relationship between the initial velocity of the hydration reaction catalysed by each isoenzyme and the initial substrate concentration was measured after verification that the initial velocity of the reaction was in each case proportional to the concentration of the enzyme. Assays were also done in the presence of constant concentrations of acetazolamide. The results of these experiments are shown in Fig. 10.

Michaelis constants and maximum velocities were determined accurately for preparations Eryth. C and Uterus C2, but the Michaelis constant of preparation Eryth. B was twice the maximum substrate concentration, and preparation Uterus C1 did not obey the Michaelis–Menten equation under the conditions of the experiment. Preparations Eryth. C and Uterus C2 were kinetically indistinguishable and acetazolamide acted in such a way as to cause an apparent increase in the affinity of the enzyme for the substrate (Figs. 10c and 10*f*). Whether this occurred as a result of combination of the inhibitor with the enzyme–substrate complex (coupling inhibition) or whether K_m was a kinetic constant equal to k_2/k_1, and the inhibitor acted to

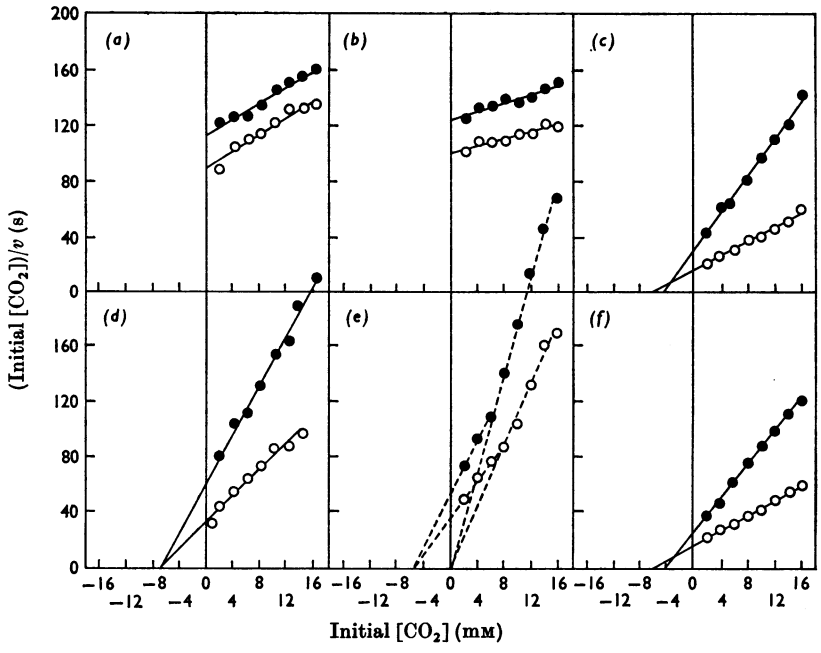

Fig. 10. Relationship between $[CO_2]$ and initial velocity of hydration of CO_2 catalysed by the carbonic anhydrase isoenzymes, illustrated by plotting [initial substrate]/(initial rate) against [initial substrate]. The experiments were carried out at 0°C at pH 7.0, with 45 mM-NaCl, 5 mM-sodium phosphate and 0.01% peptone. ○, Uninhibited; ●, in the presence of acetazolamide, at the concentrations given in parentheses. (*a*) 280 nM-Eryth. B (56 nM); (*b*) 250 nM-Eryth. B (CEP) (64 nM); (*c*) 2.6 nM-Eryth. C (5.6 nM); (*d*) 3.1 nM-Eryth. C (CEP) (6.4 nM); (*e*) 1.3 nM-Uterus C1 (2.8 nM); (*f*) 3.1 nM-Uterus C2 (5.6 nM). Constants derived from these results are presented in Table 1. The lines were fitted by least-squares regression analysis. Each point is the result of a single assay. CEP denotes purification from a chloroform–ethanol extract.

decrease k_2, is a question that can only be answered by further experimentation.

Substrate inhibition may be the explanation for the behaviour of preparation Uterus C1 but it was not possible to increase the substrate concentration to the point where this could be established definitely. In fact the evidence available (Fig. 10*e*) suggests that unexceptional non-competitive kinetics were followed as the substrate concentration was increased until an abrupt change occurred; thereafter the initial velocities of both the inhibited and uninhibited reactions remained constant with further increase in $[CO_2]$.

p-Nitrophenyl acetate hydrolase (esterase activities). Under the conditions of the experiments the initial velocity of hydrolysis catalysed by each of the isoenzymes was almost proportional to the initial substrate concentration and no satisfactory estimates could be made of either K_m or $V_{max.}$ (Fig. 11). The maximum concentration of *p*-nitrophenyl acetate was limited by its low solubility (to 3 mM).

Contamination of the endometrial and uterine homogenates with residual blood and the identity of the C2 component. Blood contamination of the tissue extracts was unavoidable with the homogenization technique used, and erythrocyte carbonic anhydrase undoubtedly contributed to the total enzyme activity extracted. Blood carbonic anhydrase in the homogenates was determined by measuring the total haematin in the tissue extracts and making the assumption that this was haemoglobin from disrupted erythrocytes. The amount of enzyme associated with this amount of haemoglobin was then calculated. Only the high-activity erythrocyte isoenzyme was determined and no attempt was made to seek the low-activity form in the tissue homogenates.

The results of several experiments, summarized in Table 2, suggest that blood enzyme accounted for most, if not all, of the carbonic anhydrase activity of non-pregnant endometrium, about 20% of the activity of the 6-days-pregnant endometrium and between 30 and 40% of the activity of the 6-days-pregnant uterus. The similarity in the last two cases between the blood-enzyme contributions to the total activities and the fractions of these

contributed by isoenzyme C2, together with the fact that preparations Eryth. C and Uterus C2 were indistinguishable electrophoretically and kinetically, suggest that the C2 form was mainly, if not completely, isoenzyme Eryth. C resulting from blood contamination. The presence of a greater amount of the C2 form in the uterine homogenate in comparison with the endometrial homogenate can also be explained in these terms; although the amount of blood in the whole uterus was similar to that in the endometrium separated from the rest of the uterine wall the total concentration of enzyme was diminished as a result of dilution by the enzymically inactive myometrial tissue.

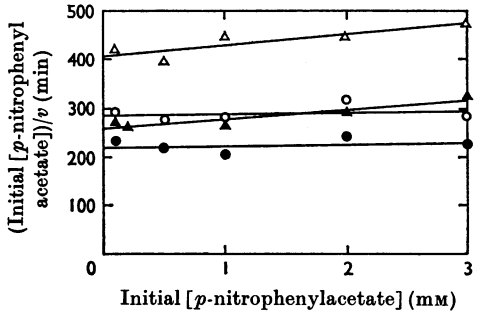

Fig. 11. Relationship between [*p*-nitrophenyl acetate] and initial velocity of the esterase reaction catalysed by the carbonic anhydrase isoenzymes. The results are illustrated by plotting [initial substrate]/(initial rate) against [initial substrate]. The experiments were carried out at 25°C at pH 8.0 with 10mM-diethylmalonate and 6% (v/v) acetone. ▲, 300nM-Eryth. B; ○, 46nM-Eryth. C; △, 30nM-Uterus C1; ●, 61 nM-Uterus C2. Each point is the average of two assays.

DISCUSSION

It is clear that the two major isoenzymes of rabbit erythrocytes, which are present in haemolysates in almost equal amounts, differ greatly in their efficiencies as catalysts. Under the conditions of the assay and with a concentration of CO_2 calculated to be sufficient to saturate both isoenzymes, Eryth. C would be 140 times as efficient as Eryth. B. To saturate Eryth. B would require 200–300mM-CO_2, but the maximum concentration of the gas in water at 37°C is limited by its solubility to about 40mM. The efficiency differential between the two isoenzymes is still greater at lower CO_2 partial pressures; with a substrate concentration of 1mM-CO_2 the differential is 500 times. Isoenzymes of carbonic anhydrase with less pronounced differences in activity have been found in the erythrocytes of human beings (Nyman, 1961; Rickli & Edsall, 1962; Laurent *et al.* 1962), rhesus monkeys (Duff &

Table 2. *Measurement of the carbonic anhydrase activity arising from residual blood contaminating the endometrium and uterus and the distribution of the total activity between the C1 and C2 isoenzymes*

For the purpose of these experiments the carbonic anhydrase activity of rabbit blood was measured in samples of whole blood from eight female rabbits. The average value was 17000±1000 (S.E.M.) units/ml of whole blood. Measurements of the amounts of blood contaminating the endometrial and uterine homogenates were made as described in the Materials and Methods section and the results are expressed as a factor giving the degree of dilution of haemoglobin in the homogenates in comparison with whole blood. The errors are expressed as S.E.M. and the numbers of observations are given in parentheses. (*a*) and (*b*) Haemoglobin contents were determined in the present work but the activities were taken from Lutwak-Mann & McIntosh (1969). (*c*) Haemoglobin content measured in (*b*) is assumed but the activity is that of the preparation from which isoenzymes Endomet. C1 and C2 were isolated. (*d*) Both the haemoglobin and activity measurements were made on the preparation from which isoenzymes Uterus C1 and C2 were isolated.

	Haemoglobin content of tissue extract (factor of dilution in comparison with whole blood)	Total enzyme activity of tissue extract (units/g wet wt. of tissue)	Contribution by blood to total enzyme activity of tissue extract (units/g wet wt. of tissue)	Blood enzyme contribution as fraction of total (%)	Amount of the C2 isoenzyme as fraction of the sum of the C1 and C2 isoenzymes (%)
(*a*) Non-pregnant endometrium	47±4 (4)	370±40 (9)	360±50 (4)	~100	—
(*b*) 6-day-pregnant endometrium	51±5 (4)	1800±160 (6)	330±50 (4)	18	—
(*c*) 6-day-pregnant endometrium	(51)	2080	(350)	17	20
(*d*) 6-day-pregnant uterus	(53)	970	320	34	37

Coleman, 1966) and certain other monkeys (Tashian, Shreffler & Shows, 1968), horses (Furth, 1968) and rats (McIntosh, 1969). It is notable that, as was the case with the high- and low-activity isoenzymes of rat erythrocytes (McIntosh, 1969), the rabbit isoenzymes show reversed efficiency as catalysts of β-naphthyl acetate hydrolysis. At a substrate concentration of 1 mM and with the conditions of the assay preparation Eryth. B was 13 times as effective as preparation Eryth. C. The nomenclature adopted for the rabbit isoenzymes is consistent with the system suggested by Furth (1968).

It is possible that the minor forms of carbonic anhydrase generated during purification were derived from the 'parent' isoenzyme by the hydrolysis of amide groups, which is thought to be the explanation for the similar behaviour of the human erythrocyte isoenzymes (Funakoshi & Deutsch, 1968, 1969). These reports reveal that incubation at low temperatures and pH 11 or above caused the release of ammonia from the human carbonic anhydrase leading to progressive formation of a series of components, the electrophoretic mobilities of which were related in a simple manner. However the enzymic activities, the zinc, nitrogen and amino acid contents and molecular weights of the members of a given series were similar (Funakoshi & Deutsch, 1969).

The effect on the kinetic properties of the erythrocyte carbonic anhydrase isoenzymes caused by treating the haemolysate with chloroform and ethanol to remove haemoglobin was similar in one respect to the experience with the rat erythrocyte enzyme (McIntosh, 1969), i.e. the K_m value of the low-activity isoenzyme was increased. On the other hand, though the K_m value for the high-activity isoenzyme was unchanged the unusual action of acetazolamide was abolished and $V_{max.}$ was halved.

Evidence suggests that the C2 isoenzyme isolated from the endometrium arises from residual blood contamination, but in contrast with this a characteristic carbonic anhydrase isoenzyme, the C1 component, is absent from blood and is found in the endometrium on day 6 of pregnancy. An increase in the content of the latter isoenzyme alone may be responsible for the increase that has been observed in carbonic anhydrase activity of the progestational endometrium in comparison with the non-pregnant one (Lutwak-Mann, 1954; Lutwak-Mann & McIntosh, 1969).

However, the possibility cannot at present be excluded that the C2 isoenzyme, which is identical with the erythrocyte high-activity form on the basis of the tests made here, can be synthesized in the endometrium. It would be interesting to test endometrial tissue from non-pregnant rabbits for the presence or absence of the C1 component, which, according to the hypothesis developed here, should occur only in pregnancy. This would be difficult because the amount of enzyme in non-pregnancy is low and the endometrium is sparse. Another important test would be to demonstrate the absence of the C1 isoenzyme in blood-free endometrial tissue from an animal on day 6 of pregnancy. This must await either the perfection of a perfusion technique capable of removing all blood from the capillary bed of the endometrium or the application of an efficient method of homogenization of this intractable tissue that preserves the integrity of the erythrocytes it contains and prevents the release of the erythrocyte isoenzymes.

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