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Genetic Variation in the Carbonic Anhydrase Isozymes of Macaque Monkeys

III. Biosynthesis of Carbonic Anhydrases in Bone Marrow Erythroid Cells and Peripheral Blood Reticulocytes of *Macaca nemestrina*[1]

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The carbonic anhydrase isozymes (CA I and CA II) of the pig-tailed macaque, *Macaca nemestrina*, have been chosen to study the regulation of enzyme levels in red blood cells. Two quantitative variants of CA I that are ideal for studies of enzyme regulation exist in this species. One variant is one of four known electrophoretic types of CA I, designated CA Ia, which is present at levels about 30% of those of the other electrophoretic types. The other is a deficiency variant of CA I which, in homozygotes, reduces the product of the CA I locus about 5000-fold and reduces the product of the CA II locus by about 60%. L-[^{14}C]Serine was used to study the biosynthesis of CA I and CA II isozymes in the reticulocytes of animals carrying these CA I variants. Specific radioactivity and total incorporation data from bone marrow erythroid cells, and peripheral blood reticulocytes indicate that the reduced CA Ia concentration is probably the result of degradation. This degradation appears to occur for only a short time before the reticulocytes enter the peripheral blood. It was not possible to determine whether the 5000-fold reduction of CA I in the CA I-deficient animals is due to reduced transcription, reduced translation, or degradation. The effect of the CA I-deficiency mutation on the synthesis of CA II was also studied. For each dose of CA I-deficiency gene, there appears to be a 30% reduction in the rate of L-[^{14}C]serine incorporation into CA II, thereby accounting for the reduced CA II concentration in CA I-deficient animals.

The *in vitro* biosynthesis of proteins in reticulocytes was first demonstrated by Borsook *et al.* (1). Since that time, most of the relevant work in this area has dealt with investigations on the biosynthesis of hemoglobin. Studies of mechanisms involved in the regulation of other red cell proteins, particularly enzymes, in mammals have been hampered by the lack of protein systems which exhibit both a sufficient number of mutations which result in altered protein

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concentrations and the potential for product analysis at the molecular level. However, with the recent demonstration of the *in vitro* synthesis of one of the carbonic anhydrase isozymes in human reticulocytes (2, 3), it is now feasible to undertake studies on the synthesis of carbonic anhydrase isozymes in primates. Carbonic anhydrase, which occurs in most mammalian erythrocytes in concentrations second only to hemoglobin, is usually present in two molecular forms, or isozymes, which have been designated CA I (or CA B) and CA II (or CA C) (*cf.* 4). Comparative sequence data (5) and genetic and evolutionary studies (6, 7) indicate that these isozymes are products of different autosomal genes which arose by gene duplication. In addition, recent studies have shown that the *CA I* and *CA II* gene loci are probably linked in rodents (8) and primates (9).

The carbonic anhydrases of the pig-tailed macaque, *Macaca nemestrina*, are ideally suited for studies of the regulation of enzyme levels in a primate species because of the considerable number of electrophoretic and quantitative genetic variants which are now known for their two carbonic anhydrase isozymes. In this species of macaque monkey, there are four types of CA I and two types of CA II which are electrophoretically distinct and are the products of the structural alleles designated *CA* I^a, CA I^b, *CA* I^c, and *CA* I^d at the *CA I* locus and *CA* II^{a_2} and *CA* II^b at the *CA II* locus (6). The *CA* I^a allele product is present at levels about 30% of the other electrophoretic types (10). In addition to the alleles determining the different electrophoretic types of CA I, there is also an inherited deficiency of CA I which results in about a 5000-fold reduction of this enzyme in red cells. This deficiency mutation is inherited codominantly and is either an allele at the *CA I* locus or closely linked to it (6). Associated with this deficiency phenotype, there is a 60% reduction in the concentration of CA II (10, 11).

The present report describes methods capable of analyzing the biosynthesis of not only CA I and CA II, but also their different electrophoretic types. By these methods it has been possible to obtain further insight into the genetic mechanisms involved in the regulation of the red cell carbonic anhydrases.

EXPERIMENTAL PROCEDURES

Materials

Uniformly labeled L-[^{14}C]serine (>100 mCi/mmole) and a standard solution of ^{14}C in toluene (4.36×10^5 dpm/ml) were obtained from New England Nuclear, and unlabeled amino acids from Sigma. TPCK[6]-treated trypsin was obtained from Worthington, MEM Eagle Vitamins 100× and Spinner Balanced Salt Solution 10× from BBL (Division of Bioquest), and cellulose polyacetate electrophoresis strips (2.5 × 15 cm) from Gelman Instrument Company. Dibutyl and dimethyl phthylate esters with specific gravities of 1.0465 and 1.189, respectively, at 25°C were obtained from Eastman Organic Chemicals. Other chemicals were reagent grade.

Preparation of Reticulocytes

Reticulocyte-rich blood was obtained from both pig-tailed macaques (*Macaca nemestrina*) and rhesus macaques (*Macaca mulatta*). Reticulocytosis was produced by daily intraperitoneal injection of a solution of phenylhydrazine hydrochloride (50 mg/ml) titrated with 1 M NaOH to pH 7.4. A dose of 7 mg/kg was given for 7 days. On Day 9, 50–80 ml of blood was drawn from a femoral vein, heparinized, and placed in ice. The hematocrit was 18–22 and the reticulocyte count was 50–95%. The blood was washed four times by centrifugation (2000*g* at 4°C) in a balanced salt solution (NKM) containing 0.153 M NaCl, 0.005 M KCl, and 0.005 M $MgCl_2$.

Preparation of Erythroid Cell-Rich Bone Marrow

Erythroid cell-rich bone marrow was produced by seven daily injections of phenylhydrazine as above. From Day 3 through Day 8 of treatment, a daily injection of 1 mg of folic acid and a daily oral dose of 100 mg of ferrous sulfate was given. On Day 9, bone marrow aspirations were performed as described by Switzer (12). To obtain a large enough sample of bone marrow for incubation, it was necessary to aspirate both ischial tuberosities and both iliac crests. The marrow samples were then treated in an identical manner as that described for reticulocyte-rich blood.

[6] Abbreviations used are: carbonic anhydrase I, CA I; carbonic anhydrase II, CA II; TPCK, L-(1-Tosylamido-2-phenyl) ethyl chloromethyl ketone: MEM, Eagle's minimum essential medium.

Incubation Procedure

Immediately after washing, the packed cells from the peripheral blood or the bone marrow were added to the incubation medium in a ratio of two parts of cells to three parts medium. The incubation medium, which contains no anemic plasma, was prepared as described by Boyer *et al.* (13). L-Serine, to be added in radioactive form, was omitted from the ^{12}C-amino acid mixture. The incubation mixture was distributed into Erlenmeyer flasks and placed in a water bath with shaker at 37°C. After 15 min of preincubation, 10 μCi of uniformly labeled L-[^{14}C]serine was added per ml of packed cells. Incubations were terminated by the addition of 4 vols of ice-cold NKM at the indicated times. The cells were then isolated by centrifugation (2000*g* for 10 min at 4°C).

Separation of Bone Marrow Red Cells by Cell Age

After incubation, the bone marrow red cells with different mean cell ages were separated according to their density by the method of Danon and Marikovsky (14). Dimethyl phthalate, and dibutyl phthalate esters were mixed to yield fluids with increments of specific gravity of 0.005. The incubated marrow cells were first layered on a fluid with a specific gravity of 1.085, and subsequently, they were centrifuged (12,000*g* for 15 min at 25°C) in a swinging bucket rotor. Cells with specific gravity less than 1.085 were then layered on a second fluid with a lower specific gravity and centrifuged again as described above. By this procedure, cell fractions with the specific gravities indicated in Table V were obtained. Each separate cell fraction was then lysed by the addition of 1 vol of ice-cold distilled water.

Isolation of ^{14}C-labeled Carbonic Anhydrases from Peripheral Blood Incubations

Cells from the incubation mixtures were lysed by addition of 1 vol of ice-cold distilled water. An aliquot of this lysate was removed for subsequent quantitation of CA I and CA II isozymes by means of a radioimmunosorbent assay (15). The concentrations of each carbonic anhydrase (μg carbonic anhydrase/mg Hb) determined in these lysates were later used to estimate total incorporation. The hemoglobin in the remainder of the lysate was precipitated at 4°C by addition of 0.4 vols of chloroform and 0.8 vols of 40% ethanol to 1.0 vols of lysate followed by vigorous stirring for 1 min. The hemoglobin precipitate was removed by centrifugation (10,000*g* for 30 min at 4°C) and the supernatant fluid dialyzed overnight at 4°C against distilled water prior to lyophilization to dryness. The lyophilized powder from each sample (corresponding to 1.5–2 ml of cells) was redissolved in 0.2 ml of distilled water. By this procedure, a 75- to 100-fold purification and a 10- to 15-fold concentration of carbonic anhydrase was realized. These concentrated enzyme solutions were centrifugated (3000*g* for 10 min) to remove particulate material. After centrifugation, 10 μl of each sample was added in duplicate to cellulose polyacetate membranes, and subjected to electrophoresis (room temperature) for 3 hr at 20 V/cm in a 0.09 M Tris/0.01 M borate/0.003 M EDTA buffer adjusted with 1 M HCl to pH 8.8 ± 0.01 (16). Subsequently, the membranes were stained with naphthol blue black and destained overnight. A complete separation of the various forms of carbonic anhydrase studied in this paper was obtained (Fig. 1).

Isolation of ^{14}C-labeled Carbonic Anhydrases from Bone Marrow Cell Fractions

The packed cell volume in some cell fractions was less than 0.1 ml. This volume is too small to permit chloroform–ethanol extraction as described for peripheral blood samples above; therefore, the separation of the carbonic anhydrases in all the cell fractions was performed in the presence of hemoglobin. The cell stromata were removed from the lysate by toluene treatment, and the lysate was subsequently dialyzed overnight at 4°C against distilled water prior to lyophilization to dryness. The lyophilized powder from each lysate was redissolved in a sufficient volume of distilled water to make a final hemoglobin concentration of 150–250 mg/ml. These concentrated lysates were applied to cellulose acetate strips and subjected to electrophoresis as above. The volume of lysate added to each strip was dependent on the concentration of hemoglobin in the sample.

Determination of Specific Radioactivity

The stained regions corresponding to each electrophoretic type of carbonic anhydrase were carefully cut from the membranes, placed into counting vials, and dissolved in Bray's solution. The volume of Bray's solution used was dependent on the mass of protein in the cut section. The amount of radioactivity was determined by counting in a liquid scintillation spectrophotometer. After counting for 10 min, each vial was internally standardized by addition of 20 μl of [^{14}C]toluene and recounted. The background was 20–24 cpm, the sample counts were at least 75 cpm, and the counting efficiency of the stained sample was 35–50%. The mass of protein in each vial was determined spectrophotometrically using the

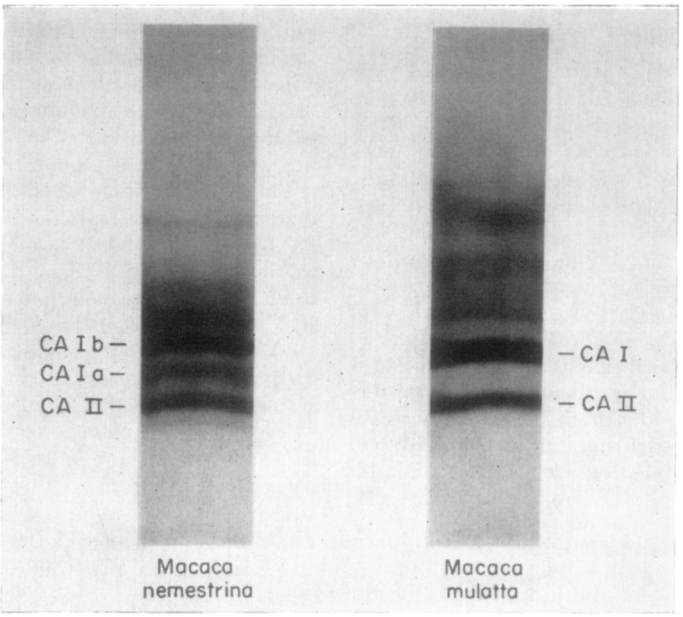

FIG. 1. Cellulose acetate electrophoresis of red cell carbonic anhydrases showing separation of CA Ia, CA Ib, and CA II of *M. nemestrina*, and CA I and CA II of *M. mulatta*. Electrophoresis (20 V/cm) carried out at room temperature for 3 hr in 0.09 M Tris/0.01 M borate/0.003 M EDTA buffer, pH 8.8.

absorbancy of naphthol blue black as a measure of protein concentration (17). The specific activity was calculated from the activity of the samples corrected to disintegrations per minute (dpm) divided by the mass of isozyme protein. The specific activities were also corrected for a difference in the number of serine residues between the CA I and CA II isozymes (17, 18).

The specific activities were standardized to 100% reticulocytes in the peripheral blood studies. This standardization was accomplished by dividing the unadjusted specific activity by the proportion of reticulocytes in the incubation mixture. The purpose of this standardization procedure was to permit comparisons between the different incubation mixtures. The hemoglobin incorporation was not used for comparison, because hemoglobin may not be synthesized during the same time period as carbonic anhydrase.

The coefficient of variation, calculated from duplicate determinations, was used to estimate the error in specific activity measurements. These relative errors were found to be 4% for CA I and 7% for CA II.

Other Procedures

Carbonic anhydrase isozymes were purified by means of column chromatography using DEAE-Sephadex (19). Monospecific antibodies towards CA I and CA II were obtained from immunized rabbits (20). The concentration of CA I and CA II (μg carbonic anhydrase/mg Hb) was determined in the whole cell lysates by a radioimmunosorbent assay (15). The isolation of peptides from a tryptic digest of enzyme was performed as described by Tashian *et al.* (21). Hemoglobin was determined spectrophotometrically as hemoglobin cyanide (22). Hemoglobin specific radioactivities were determined in a manner similar to that used for the carbonic anhydrase specific radioactivities. Five microliters of cell lysate, for which a hemoglobin concentration had been determined, were streaked on a cellulose acetate electrophoresis strip. The strip was immediately placed in naphthol blue black and destained overnight. The estimation of ^{14}C-radioactivity (dpm) was performed precisely as that described above. The specific radioactivity was then calculated as dpm/μg Hb added to the strip.

RESULTS

Evidence for Chain Initiation

The carbonic anhydrase isozymes were isolated by means of column chromatography (19) from pooled, concentrated enzyme solutions obtained from the four hour incubation studies (see Experimental Pro-

cedures). The specific activities (dpm/μg isozyme) of CA Ia, CA Ib, and CA II from *M. nemestrina* were found to be in agreement with the corresponding values obtained by means of cellulose acetate electrophoresis (Table I). The ^{14}C-labeled CA Ib was obtained in sufficient quantity (approximately 2.0 mg) to determine the amount of radioactivity in single peptides isolated by peptide mapping of a tryptic digest of the isozyme. Three different peptides were selected for the analysis: (a) the N-terminal peptide (T-1), (b) the C-terminal peptide (T-4), and (c) a peptide from the interior of the molecule (T-2). Each of these peptides contains one serine residue. In addition, a peptide (T-3) which lacks serine residues was chosen as a control. The results of the determination of C^{14}-radioactivity in these peptides are shown in Table II. As can be seen, the specific activities (dpm/residue serine) of the three peptides containing one serine residue are approximately equal.

TABLE I

L-[^{14}C]SERINE-SPECIFIC ACTIVITIES OF CA Ia, CA Ib, AND CA II FROM RETICULOCYTES OF *Macaca nemestrina* SEPARATED BY MEANS OF COLUMN CHROMATOGRAPHY AND CELLULOSE ACETATE

Carbonic anhydrase form	Specific activity (dpm/μg CA)	
	Cellulose acetate	DEAE-A50
CA Ia	38.0	36.5
CA Ib	12.1	12.4
CA II	7.3	8.0

Time-Course Studies

The incorporation of L-[^{14}C]serine into each carbonic anhydrase component was determined during 20 hr of incubation for both *M. nemestrina* and *M. mulatta* and the specific activities were plotted as a function of incubation time (Figs. 2 and 3). It is evident that there are considerable differences among the specific activities of the various forms of carbonic anhydrase.

Four-Hour Incubation Studies

The results described in Figs. 2 and 3 indicate that the incorporation of L-[^{14}C]-serine into CA I approaches maximum values after 4 hr of incubation. This 4-hr incubation period was chosen for further studies. The values for concentration, total incorporation, and specific activity obtained for each form of carbonic anhydrase in *M. mulatta* and *M. nemestrina* are shown in Tables III and IV, respectively. CA I in *M. mulatta* and CA Ib in *M. nemestrina* are present in higher concentrations than their CA II isozymes. In agreement with the lower CA II concentration, there is also a lower total incorporation of L-[^{14}C]serine

TABLE II

INCORPORATION OF L-[^{14}C]SERINE INTO SELECTED TRYPTIC PEPTIDES OF CARBONIC ANHYDRASE I ISOLATED FROM RETICULOCYTES OF *Macaca nemestrina*

	Tryptic peptide[a]	Activity (dpm)	Approximate relative yield of peptide	Normalized total activity
T-1	(Acetyl-Ala-*Ser*-Pro-Asp-Trp-Gly-Tyr-Asp-Asp-Lys) 1 5 10	495	0.5	990
T-2	(Thr-*Ser*-Glu-Ala-Lys) 35	818	0.8	1022
T-3	(Leu-Gln-Lys) 157	0	0.9	0
T-4	(Ala-*Ser*-Phe-COOH) 258 260	1145	1.0	1145

[a] Residue numbers based on a total of 260 residues for human CA I (36). Inferred sequences of tryptic peptides from Tashian and Stroup (37).

into CA II than into CA I or CA Ib. It should be noted that the specific activity of CA II in *M. nemestrina* is constant over a wide concentration range, and therefore, the total incorporation into CA II is directly proportional to CA II concentration. Unexpectedly, the CA Ia specific activity in *M. nemestrina* appears to be inversely pro-

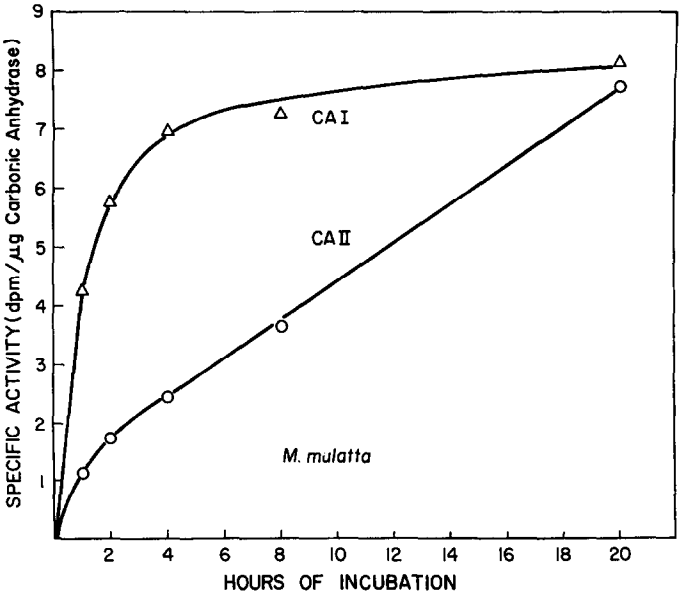

FIG. 2. Time course of incorporation of L-[^{14}C]serine into the carbonic anhydrases of reticulocytes from *M. mulatta*. △——△, CA I; ○——○, CA II.

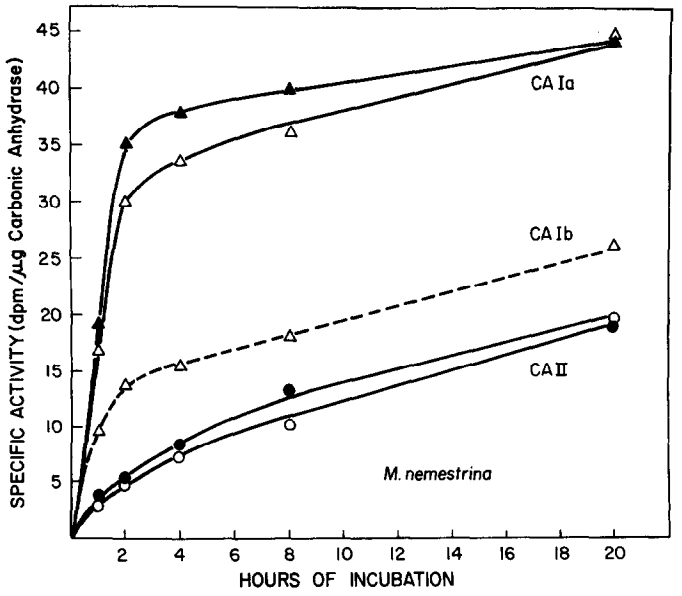

FIG. 3. Time course of incorporation of L-[^{14}C]serine into the carbonic anhydrases of reticulocytes from *M. nemestrina*. Solid triangles and circles are determinations on an animal with the *a/o* genotype. Open triangles and circles are determinations made on an animal with the *a/b* genotype. ▲——▲, CA Ia; ●——●, CA II △——△, CA Ia; △-----△, CA Ib; ○——○, CA II.

TABLE III

FOUR-HOUR INCORPORATION OF L-[^{14}C]SERINE INTO THE RETICULOCYTE CARBONIC ANHYDRASES OF *Macaca mulatta*

Animal number	Reticulocytes (%)	CA I			CA II		
		Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA I)	Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA II)
1	65	7.4	50.8	6.87	2.1	5.48	2.61
2	85	11.7	101.2	8.65	2.4	8.06	3.36

TABLE IV

FOUR-HOUR INCORPORATION OF L-[^{14}C]SERINE INTO THE CARBONIC ANHYDRASE OF RETICULOCYTES FROM THREE *Macaca nemestrina* WITH DIFFERENT CA I GENOTYPES

CA I genotype	Reticulocytes (%)	CA Ia			CA Ib			CA II		
		Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA I)	Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA Ib)	Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA II)
a/b[a]	50	3.5	134	38.2	9.5	131	13.8	3.5	29.4	8.4 IIb[b]
a/b	75	2.5	124	49.5	10.5	161	15.3	2.7	17.3	6.4 IIb
a/b	80	4.2	140	33.4	8.8	135	15.3	3.2	23.2	7.3 IIb
a/b[c]		3.4	133	40.4	9.6	142	14.8	3.1	23.3	7.4 IIb
a/o	90	2.7	103	38.0	—	—	—	1.8	15.0	8.4 IIb
o/o	70	—	—	—	—	—	—	1.0	9.3	9.3 IIb
o/o[d]	70	—	—	—	—	—	—	0.5	[4.1][e]	[8.1] IIb
		—	—	—	—	—	—	0.5	[4.4]	[8.8] IIa_2

[a] Same *a/b* animal was used for these experiments.
[b] CA II allelic type.
[c] Average of the three *a/b* incubations.
[d] This animal was heterozygous for CA II (II^{a_2}/II^{b}).
[e] Bracketed values have been extrapolated from an eight-hour incubation.

portional to the CA Ia concentration. The CA Ia incorporates L-[^{14}C]serine at the same rate as CA Ib.

Pulse-Chase Incubations

Approximately 12 ml of packed reticulocytes were incubated with L-[^{14}C]serine as described above. After 90 min, 10.5 ml of cells were removed and synthesis was interrupted by the addition of 4 vol of ice-cold NKM. The remaining 1.5 ml was incubated for an additional 90 min (for a total of 3 hr) before terminating synthesis. The 10.5 ml of cells were then collected by centrifugation (3000*g* for 10 min at 4°C), washed once in 4 vol of ice-cold NKM, and collected again by a second centrifugation step. A 1.5-ml aliquot of cells was removed at this time and treated as described above for determination of L-[^{14}C]serine incorporation. The remaining 9 ml of cells was divided equally; 4.5 ml was placed into media with L-[^{14}C]serine and the other 4.5 ml placed into medium containing a 5× normal incubation concentration of L-[^{12}C]serine. The cells were allowed to incubate further, aliquots of 1.5 ml each were removed, and synthesis terminated at the indicated times (Fig. 4).

The results of these experiments are described in Fig. 4. The effect of the medium change after 90 min is to prolong the period

of maximum synthesis. This effect can easily be seen by comparing the specific activities of the cells whose medium was changed with the specific activities of the untreated control cells. It can also be seen that the specific activities of all forms of carbonic anhydrase remain constant after the removal of L-[^{14}C]serine.

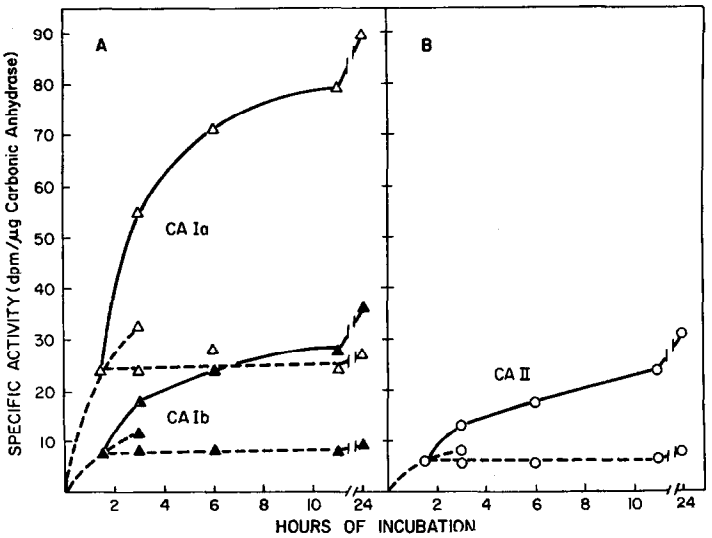

FIG. 4. Time course of L-[^{14}C]serine-specific radioactivities for (A) CA Ia, CA Ib, and (B) CA IIb in a pulse-chase incubation of reticulocytes from CA Iab animal. Reticulocytes were incubated with L-[^{14}C]serine for 90 min. An aliquot was then removed and allowed to incubate for an additional 90 min. The remaining cells were divided into two equal portions, one of which was placed into fresh medium with L-[^{14}C]serine and the other into fresh medium with L[^{12}C]serine. Both portions were then allowed to incubate further and aliquots were removed at the indicated times for analysis. Unbroken lines indicate the specific activities in cells incubated in L-[^{14}C]serine after initial pulse, the curved dashed lines are the specific activities of the control cells (3-hr incubation), and the horizontal dashed lines indicate specific activities in cells incubated in L-[^{12}C]serine after initial pulse (chase). CA Ia (△), CA Ib (▲), CA IIb (○).

TABLE V

TWO-HOUR INCORPORATION OF L-[^{14}C]SERINE INTO THE CARBONIC ANHYDRASES OF *Macaca nemestrina* BONE MARROW ERYTHROID CELLS AS A FUNCTION OF MEAN CELL AGE

CA genotype	Specific gravity of cell fractions	CA Ia			CA Ib			CA II			Hb
		Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA Ia)	Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA Ib)	Concentration (μg/mg Hb)	Total incorporation (dpm/mg Hb)	Specific activity (dpm/μg CA II)	Specific activity (dpm/μg Hb)
a/b	<1.065	4.9	2300	469.3	7.0	2528	361.1	1.8	754	419	234.3
	<1.085 > 1.065	7.6	1166	153.4	13.2	1001	75.8	3.4	291	85.7	106.5
	>1.085	2.5	21	8.3	14.7	27.2	1.9	4.0	9.9	2.4	2.6
b/o	<1.065	—	—	—	—	—	198	—	—	200.8	—
	<1.07 > 1.065	—	—	—	9.0	531	57.0	1.8	133	74.1	123
	<1.085 > 1.07	—	—	—	7.4	63.5	8.6	3.4	24.1	7.04	43.2
	>1.085	—	—	—	8.6	12.05	1.4	2.5	—	—	3.9

Bone Marrow Incubations

The values for concentration, total incorporation, and specific activity for each form of carbonic anhydrase in *M. nemestrina* bone marrow cell fractions are shown in Table V along with their respective specific gravities. The separation of cells with different mean cell ages is reflected by the specific activity measurements of both carbonic anhydrase and hemoglobin for each cell fraction. In agreement with the low CA II concentration, there is a lower total incorporation of L-[^{14}C]serine into CA II than into CA Ib in the incubation of both CA I *a/b* and CA II *b/o* marrows. It should be noted, however, that CA Ia total incorporation is equal to that of CA Ib, although the concentration ratio of CA Ib to CA Ia increases with increasing cell age. The greatest change in this ratio occurs in the oldest cell stage.

DISCUSSION

Validity of Carbonic Anhydrase Synthesis Rates Determined in Reticulocytes

One prerequisite for an interpretation of our results is the assumption that the observed rates of *in vitro* biosynthesis generally reflect the over-all rates of *in vivo* biosynthesis. However, since most protein synthesis has probably been completed before the reticulocyte stage, there may be no quantitative relationship between the *in vivo* and the *in vitro* rates of synthesis. The results of Herzberg *et al.* (23) and Gee-Clough and Arnstein (24), nevertheless, indicate that the low synthetic rate in reticulocytes is due to a generalized reduced capacity to initiate polypeptide chain synthesis rather than to a loss of mRNA. Therefore, if the reduction in the capacity for synthesis is such that the relative rates of synthesis are maintained, a proportionality would be expected between the concentration of a specific protein and the total amount of radioactive label it incorporates during *in vitro* incubation. That the concentrations of proteins synthesized in the reticulocyte, are in fact approximately proportional to their total incorporation of radioactive label has been demonstrated for hemoglobins A and A_2 in man (25), hemoglobins S and A_2 in man (26), and also for hemoglobins A and A_2 in *Ateles paniscus* (27). Data presented in this paper for CA Ib and CA II of *M. nemestrina* and CA I and CA II of *M. mulatta* are also generally consistent with the assumption that concentration is proportional to total radioactivity.

Time-Course Studies

The results of our time-course studies indicate that the CA Ia specific activity curves in CA Ia and CA Iab animals are identical, suggesting that no interaction between these two electrophoretic types occurs during synthesis.

Initial rates of synthesis of both CA I and CA II isozymes are maintained for only 1–2 hr of incubation. As demonstrated by the pulse-chase experiments, initial rates of synthesis can be maintained for an additional 1–2 hr by simply removing the used medium and replacing it with fresh medium (Fig. 4). Whether initial rates can be maintained over longer periods of time by this procedure has not been determined. Borsook *et al.* (1) and Jonxis and Nijhof (28) have shown essentially the same type of stimulation for hemoglobin synthesis. Borsook *et al.* (1) demonstrated that a boiled, nonprotein filtrate of liver would stimulate synthesis while Jonxis and Nijhof (28) observed a stimulation by dialyzed, non-heat-denatured anemic plasma. The stimulating factor(s) in our experiments, like those of Borsook *et al.* (1), are probably essential vitamin(s) and not protein as suggested by Jonxis and Nijhof (28).

Synthesis of the Carbonic Anhydrase I and II

Animals that are homozygous for the CA I-deficiency gene show a 60% reduction in CA II concentration compared to animals not carrying the deficiency, and animals heterozygous for the deficiency show a 30% reduction in CA II levels. The effect of the CA I-deficiency mutation on the CA II concentration seems to be related solely to the dose of the deficiency gene, and not to the level of CA I (10). Recent evidence indicates that the *CA I* and *CA II* loci are linked, and that the effect of the deficiency gene is to reduce the level of the CA II allelic product that is *cis*

to it by 60 % (9, 10). The total incorporation of L-[^{14}C]serine into CA II is also reduced by approximately 30 % for each dose of the deficiency gene. Moreover, the total incorporation into each CA II allelic product in a homozygous deficient animal is reduced by 60 % (Table IV). Therefore, the decrease in CA II concentration associated with the deficiency gene apparently results from regulation at the transcriptional or translational level and not at any posttranslational level. This apparent polar effect of the CA I-deficiency gene on the synthesis of CA II is currently being investigated.

CA Ia and CA Ib incorporate L-[^{14}C]serine at equal rates even though their concentrations differ 3- to 4-fold. This equal total incorporation, despite concentration differences is not consistent with the assumption that total incorporation is porportional to concentration. Since the CA Ia specific activity appears to be inversely proportional to CA Ia concentration, it is clear that the final CA Ia concentration is not determined entirely by its rate of synthesis. The CA Ib and CA II specific activities, however, are constant over the concentration ranges examined; therefore, their final concentrations are determined mainly by their rates of synthesis. The assumption of proportionality between total incorporation and concentration is generally correct, but does not take into account other factors, not related to synthesis, which can also determine final protein concentrations.

The CA Ia, Ca Ib, and CA II concentrations have been determined in young and old peripheral red blood cells which were separated by differential centrifugation (DeSimone, unpublished results). It was found that these molecules are stable and show no concentration changes during the life span of the mature erythrocyte. Therefore, the low concentration of CA Ia relative to CA Ib must result from a regulatory process which occurs at some point during the period of synthesis. Results of the time course studies demonstrated that CA Ia and CA Ib incorporated L-[^{14}C]serine at equal rates during 20 hr of incubation, and therefore indicated that degradation does not occur in the reticulocyte. To further exclude the possibility of degradation during this time period, pulse-chase experiments were performed. The rationale for the pulse chase is that the turnover rate of CA Ia during the chase period should be higher than that for CA Ib, and the difference in turnover rates should be reflected in diminished CA Ia total ^{14}C-radioactivity relative to CA Ib. Since the total ^{14}C-radioactivity remained constant for both CA Ia and CA Ib during the chase period, it can be concluded that degradation does not occur in the reticulocyte.

The finding in the bone marrow studies, that the CA Ib to CA Ia concentration ratios markedly increase with increasing cell age while the total incorporations remain approximately equal at each cell stage, clearly demonstrates that the CA Ia concentration is dependent upon its rate of degradation. This net loss of CA Ia may result from either passive denaturation or active catabolism by a proteolytic enzyme. Whatever the mechanism of degradation, it must be limited to a short time period before the reticulocyte passes into the peripheral blood since there is no evidence for degradation occurring either in the mature red cell or in the older reticulocytes. Experimental evidence supports the idea that turnover of nonhemoglobin protein is rapid during the earlier cell stages of erythrocyte development (29, 30).

Itano (31) proposed that differential survival of completed hemoglobin molecules may be an important factor in determining the final concentration of human variant hemoglobins. Since that time, a number of hemoglobins have been characterized which have abnormal heat stabilities and low cellular concentration (32). The most recent evidence in support of degradation has come from synthesis studies of a heat-unstable hemoglobin, Hb Ann Arbor (33). Their evidence indicates that newly synthesized abnormal α-chains are most susceptible to degradation.

Degradation has also been implicated as a mechanism for the regulation of protein concentrations in many mammalian tissues other than blood (34, 35).

Experimentally, it is difficult to distinguish whether the low level of CA I present in CA I-deficient animals is due to a syn-

thetic defect or to degradation. If degradation is responsible for the low CA I concentration, it would have to proceed at approximately the same rate as that of synthesis. Assuming degradation occurs randomly in the free CA I pool, incubation of deficiency reticulocytes should produce CA I with a markedly increased specific activity relative to the nondeficiency CA I electrophoretic types. No incorporation of L-[^{14}C]serine into CA I could be demonstrated, although approximately 2.0 μg of purified CA I was analyzed. Therefore, either CA I in these animals is preferentially lost as newly forming or newly formed molecules, or the synthesis of CA I is proceeding at a 5000-fold reduced rate. Unfortunately, it was not possible to examine the polyribosomes.

One additional observation which requires more experimental evidence is that carbonic anhydrase and hemoglobin may be made during different time periods in erythrocyte development. By comparing the relative change in the specific activity of hemoglobin with that of CA Ib or CA II in bone marrow, it appears that hemoglobin synthesis falls off less rapidly than carbonic anhydrase synthesis. Since the concentrations of CA Ib and CA II relative to hemoglobin are low in the early cell stages, it is possible that hemoglobin synthesis starts prior to, and ends later than, carbonic anhydrase synthesis.

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