techniques, while others (*10*) have investigated some of the many organic products ultimately produced. Their observations have been applied to further studies (*11, 12*) of the photochemistry of tryptophan incorporated in protein and the resulting effects on nucleic acids.

We now report the identification of one of the biologically active products resulting from the photooxidation of tryptophan in aqueous solution as hydrogen peroxide (H_2O_2). This compound accounts for some of the specific biological effects mentioned above: selective killing of *recA* mutants, mutagenicity, and others.

Photolysis of an oxygenated, aqueous solution of tryptophan for 24 hours, with lamps that emit maximally at 350 nm, produces a crude mixture that is selectively toxic to *recA* bacterial mutants. A starch-iodide qualitative test of this mixture demonstrated the presence of an oxidant, and thin-layer chromatography (TLC) indicated that H_2O_2 was present. Chromatographic purification (followed by bacterial killing assay) of the biologically active material [tryptophan photoproduct (TP)] on Sephadex G-10 afforded partially purified TP, which could be further separated from other photoproducts by ion exchange chromatography on Sephadex QAE resin. Final purification was accomplished by chromatography on a second G-10 column (Table 1).

Investigation of the possibility that TP is H_2O_2 was carried out by comparison of the spectrometric, chromatographic, chemical, and biological properties (see Table 2). Thus, both TP and H_2O_2 show no significant ultraviolet absorption other than "end absorption," increasing from about 230 nm. Both behave identically on cellulose TLC plates with two different solvent systems, and on silica gel TLC plates with one system, in addition to having identical Sephadex G-10 elution volumes; both give positive starch-iodide, lead tetraacetate, and *p-N,N*-dimethyldiaminobenzene tests, and both are reduced by sodium borohydride; finally, both are destroyed by catalase and show approximately 64-fold greater toxicity to *recA* mutants than to an isogenic *recA*$^+$ control. These results provide confirmation that the biologically active material TP is H_2O_2.

Semiquantitative analysis indicated that the crude photomixture, after irradiation for 24 hours, has an H_2O_2 concentration of as much as 0.015*M*, compared with an initial tryptophan concentration of 0.05*M*, indicating a 30 percent yield based on organic material. This is in line with the observations of Wood (*13*), who studied H_2O_2 production from the dye-sensitized photooxidation of amino acids, and of Santus (*11*), whose results indicated that the primary photooxidation product of tryptophan (*N*-formylkynurenine) is a good sensitizer. The H_2O_2 detectable by starch-iodide and *p-N,N*-dimethyldiaminobenzene is present after 10 minutes; and H_2O_2 can be detected by a bacterial killing assay after 40 minutes.

In view of these results, the genetic effects of H_2O_2 take on added importance. At least some of the above-mentioned biological properties, in addition to selective *recA* bacterial killing and mutagenicity, presumably are due to H_2O_2. The mechanism giving rise to such relatively large amounts of H_2O_2 is also of considerable interest (*14*).

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14. We also have found H_2O_2 to be produced on photooxidation of the tryptophan-rich enzyme lysozyme. Under the same conditions employed for tryptophan photolysis, H_2O_2 can be detected after 18 hours, and when the photolysis was carried out with added *N*-formylkynurenine, H_2O_2 was detected after only 4 minutes.
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Inhibition by Anions of Human Red Cell Carbonic Anhydrase B: Physiological and Biochemical Implications

Abstract. *The hydration rate of CO_2 catalyzed by human red cell carbonic anhydrase B is 92 percent reduced by the normal concentrations of chloride and bicarbonate in red cells. This reflects a general sensitivity of this reaction to halides and other anions, up to 87 times greater than the effect on red cell carbonic anhydrase C. The catalytic hydration of CO_2 is generally more (up to 24 times) sensitive to inhibition by anions and sulfonamides than the dehydration of HCO_3^-, probably reflecting different mechanisms. The sensitivity of enzyme B to anion inhibition also depends upon the substrate, being much greater for CO_2 than for certain esters. On the basis of the very low catalytic activity of B for CO_2 in the presence of physiological concentration of chloride, and the fact that carbonic anhydrase C is effective for CO_2 hydration (in the presence of chloride) at a rate 340 times greater than that of CO_2 output from tissues, it appears that the biological role of enzyme B is not that of a carbonic anhydrase.*

There are two zinc enzymes of similar size (molecular weight, 30,000), shape (spherical, cleft to active site, single chain, low helix), amino acid sequence, and active site structure in human red cells. Both catalyze the reversible hydration of CO_2 and the hydrolysis of certain esters. Because of the first of these properties, they are both called carbonic anhydrase (carbamate hydrolase, E.C. 4.2.1.1) but their characteristics are quite different (*1–5*).

Separation from blood by electrophoresis yielded 4 g of carbonic anhydrase B per liter of red cells, and 0.7 g of carbonic anhydrase C per liter of red cells. Several significant points have emerged: (i) The turnover number of C for CO_2 or HCO_3^- at *p*H 7.4 is some 20 times greater than that for B. (ii) With certain esters as substrate, B is more active than C (*1*). (iii) The activity and general properties of most tissue carbonic anhydrases closely resemble those of C. Kidney and stomach enzyme are of this high activity type, and are inhibited by $\sim 10^{-8}M$ acetazolamide (*2*). Carbonic anhydrase B and low activity enzyme from the blood of the rat, the rabbit, and the guinea pig, from the uterus and intestinal tissues of some of these species, and from the liver of the male rat required 10 to 1000 times as much acetazolamide (*6, 7*). (iv) Bovine and canine red cells contain but one carbonic anhydrase; each resembles C with regard to high activity and the inhibitory effect of acetazolamide and congeners at 10^{-8} to $10^{-9}M$ (*2, 3*). (v) Chemical differences between B and C suggest differing functions. In the dehydration reaction, the Michaelis constant (K_m) of C rises with *p*H, consistent with HCO_3^-

Table 1. Effect of anions upon hydration and dehydration of CO_2 catalyzed by human red cell carbonic anhydrases B and C. For hydration, inhibitor and enzyme were mixed for 2 minutes to ensure equilibrium. The mixture is added to the CO_2 solution, and the reaction was started by addition of buffer. This procedure was designated EI (*13*); in the SEI procedure, enzyme and inhibitor were incubated in the presence of substrate (CO_2). Under present conditions, the two procedures give identical inhibition data. For dehydration, enzyme, inhibitor, and buffer were incubated for 2 minutes, and the reaction was started by the addition of $NaHCO_3$. The numbers given are means of at least five determinations. The results are presented as millimolar concentrations yielding 50 percent inhibition.

Inhibitor	Hydration*		C/B	Dehydration†		C/B
	B	C		B	C	
NaF, KF	> 300	> 300		370	420	1
NaCl	6 (15)	200	33	105	600	6
NaBr	4	200	50	54	600	11
NaI	0.3 (0.6)	26	87	6	115	19
$NaHCO_3$	12	70	6			
Na_2HPO_4	3	36	12	> 100	> 100	
Sodium acetate	7	70	10	165	132	1
$NaNO_3$	7	35	5	82	90	1
$KClO_4$	3.6	1.3	0.3	14	5	0.3
KCNS	0.2	0.6	3	1.6	4.6	3
KCNO	7×10^{-4}	0.02	28	8×10^{-3}	0.12	15
Acetazolamide	2×10^{-4}	6×10^{-6}	0.03	2×10^{-4}	2×10^{-5}	0.1

*The method is described in (*13, 14*); 7 m*M* barbital buffer; 5 m*M* CO_2; enzyme concentrations, 3×10^{-8} *M* (B) and 4×10^{-9} *M* (C). Numbers in parentheses indicate that the reaction was performed in 7 m*M* phosphate buffer. †9 m*M* phosphate buffer; 30 m*M* HCO_3^-; enzyme concentration was 4×10^{-8} *M* (B) and 2×10^{-9} *M* (C).

reacting with the acid form of the enzyme, but B shows no change of K_m with *p*H (*8*). There are differences in reactivity; certain alkylating sulfonamides and iodoacetate irreversibly inhibit B, but not C (*9*), and imidazole also inhibits B but not C (*10*).

These facts encourage speculation that B may have a physiological function unrelated to the CO_2 system. With regard to point (i) above, we have calculated the net velocity of the reaction $HCO_3^- \rightarrow CO_2$ in the lung capillaries, taking into account the kinetic properties of enzyme C, its molar concentration in blood, and the appropriate volume in which the exchange occurs. This yields a net rate of 1460 mmole/min. The equivalent calculation for the reaction $CO_2 \rightarrow HCO_3^-$ in tissue yields 3040 mmole/min. In resting man, CO_2 output is 12 mmole/min, so that there is an excess of 120-fold for dehydration and 280-fold for hydration. At maximum exercise, the excess is 34- and 61-fold, respectively. These data suggest no physiological need for a second carbonic anhydrase of lesser activity (*11*).

Our interest in the role of anions in this problem was kindled by Wistrand and Lindahl's (*12*) measurements of human red cell and renal carbonic anhydrase at 37°C and *p*H 7.1. They reported that the inhibition constant (K_I) for Cl^- against B was 28 m*M* and against C it was > 200 m*M* in both hydration and dehydration reactions. This was close to Carter and Parsons' (*7*) data for chloride inhibition of hydration at 0°C, with the low (K_I = 40 m*M*) and high (K_I = 400 m*M*) activity enzymes from guinea pig red cells. These workers (*4, 7*) have also reviewed the earlier literature on anions. Magid has reported K_I for Cl^- against dehydration catalyzed by B (at 1.5°C) as 38 m*M* at *p*H 6.8 and 70 m*M* at *p*H 7.4. There was no effect on C in this concentration range. This moderate degree of inhibition upon B by Cl^-, and much less effect on C appeared of chemical but little physiological interest. We had found that potassium chloride at 143 m*M* had no inhibitory effect on canine red cell carbonic anhydrase (a typical high activity enzyme), at 37°C and 3 m*M* CO_2 (*13*). This agreed with the weak activity of Cl^- on the bovine red cell and other high activity enzymes. We have now made five sets of observations on the human enzymes.

1) In the hydration reaction enzymes B and C are inhibited by chloride. The enzymic hydration of CO_2 was studied as described (*14*) and is indicated in Table 1. Barbital was used as buffer in this and our earlier work, as we have consistently found it to be noninhibitory. The initial *p*H was 7.6 and the final *p*H was 6.9. The rate of formation of H^+ was linear, reflecting constancy of CO_2 concentration and the independence of the hydration rate constant on *p*H. The enzymic reaction was also linear (*10*). We report I_{50} values (concentration that reduces enzymic rate by half) rather than K_I because the mechanism is not certain for all of these substances. In these experiments $I_{50} > E$ (enzyme concentration); and $K_m \geqq S$ (substrate concentration; since K_m is 9 m*M* for C and 4 m*M* for B (*10*). Thus for either noncompetitive or competitive inhibition, I_{50} does not differ greatly from K_I.

The I_{50} for Cl^- against B was 6 m*M*, much lower than found by others (*7, 12*). At the concentration of chloride in red cell water (about 80 m*M*) the enzyme is 92 percent inhibited (Fig. 1). This is also the theoretical relation between I_{50} and 92 percent inhibition (about 13 times greater) for a simple biomolecular reaction between enzyme and inhibitor noncompetitive with substrate. From this finding it appeared unlikely that B could function as a primary catalyst for CO_2 under physiological conditions. However, attention was also given to the effect of HCO_3^-, whose concentration in red cells is about 15 m*M*. As would be expected, it had a strong effect on B, both inhibiting hydration (Table 1) and competing with the effect of Cl^-. The latter is manifest by reduction of Cl^- inhibition at 85 m*M* from 93 to 72 percent. However, since both ions inhibit, the net effect of a solution of 85 m*M* Cl^- + 15 m*M* HCO_3^- is to reduce the initial activity of B to about 8 percent of normal. Inhibition of C by chloride is negligible (Table 1).

Our low value for I_{50} for Cl^- against B, compared to those cited (*7, 12*), is due to our use of a noninhibiting and noncompeting buffer. In earlier studies (*7, 12*) 20 to 25 m*M* phosphate was used; and, since its I_{50} is about 3 m*M* (Table 1), all I_{50} data for anions or drugs would be affected by a factor of about 8, assuming competition. If we make the appropriate correction for the observed I_{50} or K_I of 40 m*M* (*7*) or 28 m*M* (*12*) we come close to our value of 6 m*M*. Table 1 also shows that 7 m*M* phosphate buffer raises the I_{50} of halides two- to threefold, in accord with theory.

2) In the hydration reaction, there is moderate to high inhibition against B for all the anions studied, except F^- (Table 1). Iodide and thiocyanate are highly active, and cyanate is (at 0.7 μ*M*) almost as active as acetazolamide.

3) The ratio of I_{50} of halide anions in the inhibition of C to that of B is of the order of 50. The ratios for the other inhibitory anions (excluding perchlorate) varied from 3 to 28. In striking contrast, the ratio for acetazolamide was 0.03, and those of other sulfonamides (sulfanilamide and ethoxzolamide, not shown) were less than 1. Perchlorate shows anomalous behavior to the anions, and resembles sulfonamides in this respect.

4) When *p*-nitrophenyl acetate was substrate for B, the I_{50} for Cl^- was 54 m*M*, nine times greater than for CO_2 as substrate (Fig. 1). The concentration of *p*-nitrophenyl acetate (1 m*M*) was less than its K_m (6 m*M*) (*15*) so that $I_{50} \cong K_I$. Our inhibition data for Cl^- agree with those of Verpoorte *et al.* (*15*), but differ from those of Whitney, who found K_I to be 15 m*M* (*16*). The differences in procedure included the use of different buffers. With *o*-nitrophenyl acetate as substrate, the I_{50} for Cl^- was 50 m*M*, similar to that of the paraisomer. The I_{50} of Br^- and I^- in the *p*-nitrophenyl acetate hydrolysis was 28 m*M* and 3 m*M*, respectively [very close to (*15*), but higher

than (*16*)], or 7 and 17 times greater, respectively, than found for the CO_2 reaction (Table 1). Most significant of all, however, was that Cl^- had no inhibitory effect (up to 180 m*M*) against B when 2-hydroxy-5-nitro-α-toluene sulfonic acid sultone was the substrate (Fig. 1). Bromide gave an I_{50} of about 150 m*M*, about 40 times greater than for CO_2 as substrate; iodide yielded an I_{50} of 18 m*M*, 60 times greater than for CO_2 as substrate (Table 1). This sultone is of interest in that it has the highest turnover number of any of the artificial substrates yet studied (*3*) and bears some structural resemblance to the sulfonamide inhibitors.

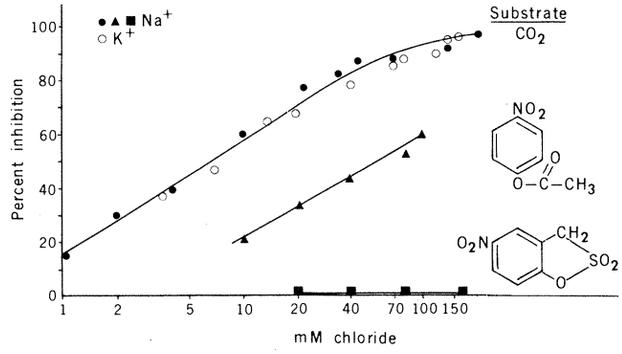

Fig. 1. The effect of chloride on reactions catalyzed by human red cell carbonic anhydrase B at 25°C. For CO_2, the method is described in Table 1 and in (*14*). For the other two substrates, see (*3*). *p*-Nitrophenyl acetate was 1 m*M*, *p*H 8; tris buffer was 7 m*M*; the enzyme was 0.3 μM, and the wavelength was 348 nm. The 2-hydroxy-5-nitro-α-toluene sulfonic acid sultone was 50 μM, *p*H 7.5; tris buffer was 10 m*M*, the enzyme was 0.07 μM, and the wavelength was 320 nm.

5) For the inhibition of the dehydration reaction $HCO_3^- \rightarrow CO_2$, a method analogous to that used for hydration was devised. A sodium bicarbonate solution was added to phosphate buffer (*p*H 6.95) which was continuously flushed with N_2 through a sintered glass plate. Enzyme and inhibitor were added to the buffer solution for equilibration. The reaction was started by the addition of $NaHCO_3$. The *p*H rise to 7.5 was monitored by a glass electrode. Under these conditions, HCO_3^- was virtually constant (falls $\sim$ 10 percent); the product was removed immediately, and the observed time for the uncatalyzed reaction (23 seconds) was in agreement with that calculated from the known rate constant (at the mean *p*H 7.2). The enzymic rate was linear with added enzyme, in the range studied, and was two to four times the uncatalyzed rate. It was not possible to use barbital in the dehydration as in the hydration because of excessive foaming. We found that up to 30 m*M* phosphate does not inhibit B or C for dehydration, in agreement with (*17*).

The compounds studied were less active in inhibiting dehydration than hydration. The single exception was acetazolamide against B. The I_{50} ratios for dehydration to hydration are 2 to 8 for C and 4 to 24 for B (excluding acetazolamide). Generally, sulfonamides are like anions in requiring more drug to inhibit dehydration than hydration (not shown). To our knowledge this fundamental comparison has not been made previously since the two systems have not been used together under reasonably similar conditions. Variation in techniques and enzyme sources appears to have obscured the situation (*2*). It is not entirely clear whether anion inhibition is competitive or noncompetitive (*8*), but this would not influence our interpretation of degree of inhibition more than twofold. For competition, since K_m is 68 m*M* for C and 32 m*M* for B (*1*), and HCO_3^- is 30 m*M*, K_I is 2/3 or 1/2 I_{50}, respectively, for the two enzymes. For noncompetition, $K_I \cong I_{50}$, since $E < I_{50}$. The greater sensitivity to inhibition in the hydration reaction may reflect the inability of the uncharged substrate, CO_2, to displace the anion, while this is possible for HCO_3^-. Our finding of different inhibition constants between hydration and dehydration would only violate considerations of equilibria, that is, the Haldane relation, if the inhibiting mechanism were the same in the two directions. This is not the case for sulfonamides (nor anions, probably) which inhibit competitively for dehydration and noncompetitively for hydration (*2, 18*). In the Haldane relation, $k_{cat}' \times K_m = k_{cat} \times K_m' \times K$, (where K is the equilibrium constant and K_m and k_{cat} have their usual meaning for hydration with primes denoting dehydration), inhibition would then leave the left side of the equation unchanged; if k_{cat} decreased and K_m' increased to the same degree, K remains unchanged as theory demands. There is work in progress on this unusual problem.

Our data suggest important differences at the active site between B and C, and show that, biologically, B is not a carbonic anhydrase. Other work supports these views. Consideration of Ward's nuclear magnetic resonance data on the broadening of the $^{35}Cl^-$ linewidth shows that, at *p*H 7.4 and 0.15*M* NaCl, B is at least five times as effective as C in binding chloride (*19*). The data of Fig. 1 may be a start in the direction of ascertaining the true physiological role of B, since we show differences in the activity of B against the several substrates tested in the presence of NaCl or KCl. It has been shown that B is a better catalyst than C for *o*-nitrophenyl acetate, the reverse being true for the para-isomer (*15*). Inhibition data also indicate specificity of substrate, since acetazolamide is 10 to 1000 times more active in inhibiting CO_2 hydration than it is in the ester hydrolyses (*2*). The affinity constant for acetazolamide and C, measured from the rate of reaction (*20*), matches its inhibition constant for CO_2 hydration against either bovine enzyme (*21*) or C (Table 1). This match would not occur if the ester function were being measured for either human C or the bovine enzyme. It is also clear that acetazolamide is a stronger inhibitor of C (or other high activity enzymes) than of B (Table 1) or other low activity enzymes (*6, 7*).

These data show that each reaction must be examined specifically for the analysis of inhibition by drugs or buffers. Different substrates or directions yield different results, illustrating the subtle nature of the active site or sites. The effect of phosphate was overlooked, and masked the magnitude of chloride inhibition, since, of the reactions studied, it only acts significantly on hydration by B. However, Whitney (*22*) obtained precisely the same K_I as ours, 3 m*M*, for phosphate inhibition of *p*-nitrophenyl acetate hydrolysis by B, which is analogous to hydration.

Since acetazolamide is used in the treatment of glaucoma (250 mg orally three or four times per day) we may ask whether any unusual effects have been reported which may elucidate the role of B in human physiology (Fig. 1). However, if the I_{50} for acetazolamide against whatever function of B may be involved is the same as for nitrophenyl acetate hydrolysis (0.4 μM) (*15*), only about 90 percent of B would be inhibited, since the concentration of acetazolamide not bound to plasma proteins and free to react with enzyme is about 1 μg/ml or 4 μM (*23*). With the high concentration of B present, the residual enzyme is probably adequate for whatever function it has and probably it has not been totally inhibited, on such a treatment schedule. However, there are a few instances in which volunteers received an intravenous dose of the order of 100 mg/kg (*24*). One such individual reported marked intoxication, ataxia, red blurred vision, and difficulty in thinking, but survived.

Concerning the possible role of B, it seems unlikely that the second most abundant protein in red cells has no function; thus we suggest two levels of inquiry. One follows from the fact that B varies in certain diseases, notably that of the thyroid

(*25*). The second follows from nature of the active site as Zn (H_2O) $\rightleftharpoons$ Zn (OH^-) (*3, 5*) and applies to the very large number of organic reactions catalyzed by acid or bases, or involving water, such as hydrolysis, enol-keto rearrangement and aldol condensations, reactions that occur in intermediary metabolism and the biosynthesis of hormones.

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Vitamin A: Not Required for Adrenal Steroidogenesis in Rats

Abstract. *Previous work supporting the vitamin A dependency of adrenal function in rats neglected to take into account a secondary effect of the deficiency, a decrease in hepatic ascorbic acid biosynthesis. Vitamin A–depleted rats maintained on a diet free of ascorbate had a decrease in the activity of adrenal 3β-hydroxysteroid dehydrogenase, and extensive adrenocortical degeneration. The use of an ascorbate supplement prevented the symptoms. The results suggest that previous evidence for direct involvement of vitamin A in steroidogenesis may have been due to the production of a secondary deficiency, a chronic scorbutic condition.*

The vitamin A dependency of steroidogenic organ function in rats has remained a controversial subject. Both adrenal (*1, 2*) and gonadal (*3*) dysfunction have been reported in hypovitaminosis A. Attempts have been made to apply this theory as an explanation for the apparent anticarcinogenic effects of vitamin A. Light (*4*) has proposed the existence of subclinical vitamin A deficiencies which result in decreased adrenocortical output. The correction of such a condition in a patient with cancer would be the equivalent of steroid therapy. In addition, Van Thiel *et al.* (*5*) have shown that ethanol interferes with testicular vitamin A metabolism, and they theorized that this could account for the occurrence of sterility in alcoholics. However, no attempt has been made to reconcile these theories with recent work that does not support the involvement of vitamin A in steroidogenesis (*6, 7*). A review of this field revealed that none of the investigators who attempted to produce a hypovitaminosis A condition (*1–3, 6, 7*) noted an important effect of hypovitaminosis A in the rat, a reduction in the hepatic biosynthesis of ascorbic acid (*8*). An examination of the diets used indicated that in those experiments where significant steroidogenic dysfunction was noted both vitamins A and C were lacking in most of the reported diets (*1–3*). In one of the experiments that failed to support the aforementioned work (*7*), significant amounts of ascorbic acid were present in the diet (*9*). In another report (*6*) showing the absence of adrenal effects in vitamin A deficiency, no vitamin C was in the reported diet; but retinoic acid was used to deplete the vitamin A stores, and was then abruptly withdrawn. The effect of this regimen on the hepatic ascorbic acid synthesizing enzyme, gulonolactone oxidase, is not known.

With these facts in mind we decided to repeat the experiments that attempted to show vitamin A involvement in adrenal function. However, we maintained groups on vitamin A– and vitamin C–free diets.

We used 50 male, weanling Long-Evans rats (Charles River, Wilmington, Mass.). To eliminate the parameter of stress, the rats were handled every day for 3 weeks before and during the experiment. The animals were housed in wide mesh steel cages which allowed their feces and urine to drop through, leaving little residue and preventing ingestion of excreta. A light schedule of 10 hours of light (from 0800 to 1800) alternating with 14 hours of darkness was maintained. This regimen permitted stabilization of daily rhythms of enzyme and hormone activity so that normal fluctuations would occur at predictable time periods. At 6 weeks of age the rats were divided into groups and the experiment begun.

Custom-formulated diets were obtained from Nutritional Biochemicals Corp. Twenty animals were designated controls (group 1), and fed a nondeficient diet that contained 20 units of vitamin A and 1 mg of ascorbic acid per gram of feed. Groups 2, 3, and 4 (each containing ten animals) were maintained on diets free of vitamins A and C (*10*); this diet in every other aspect was identical to that given group 1. Vitamin supplements were provided as follows. Group 3 received an intraperitoneal injection of 10 mg of sodium ascorbate per 100 g of body weight per day; group 4 was given 20 international units of vitamin A palmitate per 100 g of body weight per day. Groups 1 and 2 were given equivalent injections of vehicle alone. Half of group 1 and all of groups 3 and 4 were pair-fed to group 2. The remaining animals of group 1 were given free access to their diet. However, in the results that follow the data from both control groups were pooled because there were no significant differences between them.

The group in which vitamin A was replaced (group 4) was inserted primarily as a reference point for the interpretation of any morphological changes seen in the other deficient groups and as a check on the ability of the Long-Evans strain to synthesize ascorbic acid for functional needs. The dosage of vitamin A chosen for group 4 was that amount reported to produce maximum growth and longevity without appreciable liver storage (*11*). The ascorbate supplementation of group 3 was estimated from the work of Salomon and Stubbs (*12*), who found that on the average ascorbate synthesis was 6 mg per 100 g of body weight per day. However, recent work (*13*) has shown a sizable variation in the ascorbic acid requirements of a random population of guinea pigs, and since a similar variation might exist in the levels of synthesis in rats, a dose of ascorbate just in excess of the average determined rate of synthesis was used.

The body weight and the concentration of ascorbic acid in the blood (obtained from the tail vein) were determined week-