Biochemical Pharmacology, Vol. 39, No. 8, pp. 1347–1351, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

GENERATION OF VOLATILE HYDROCARBONS FROM AMINO ACIDS AND PROTEINS BY AN IRON/ASCORBATE/GSH SYSTEM

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(*Received* 27 *April* 1989; *accepted* 11 *November* 1989)

Abstract—Incubation of free, but not of peptide-bound methionine in an iron/ascorbate system resulted in ethylene generation, which was inhibited by glutathione. Leucine and isoleucine, however, when incubated in an iron/ascorbate/GSH system, released small amounts of propane and ethane, respectively. Peptide-bound leucine additionally yielded butane, as did bovine serum albumin or casein. Hydrocarbon generation from amino acids was inhibited by hydroxyl radical scavengers, but catalase and superoxide dismutase were more efficient. Additionally, ethane and propane generation in this system was optimal at pH 6.2 suggesting the involvement of protonated superoxide besides OH-radicals which attack the side chains of Leu and Ile and very probably produce carbon-centered radicals, which should abstract a hydrogen atom from the thiol group of GSH resulting in the formation of saturated hydrocarbons.

Animals regularly exhale small amounts of the volatile hydrocarbons ethane, ethylene, propane, butane and pentane [1, 2]. The amount of these gases produced is higher than that detectable in the expired air because of their partial oxidation by a cytochrome P-450 isoenzyme [2–4]. Although some investigators believe that these gases are generated by intestinal bacteria [5], this could not be confirmed in experiments in our laboratory. Various non-absorbable antibiotics and sulfonamides did not diminish the exhalation of alkanes [6].

In model experiments with erythrocytes, which were peroxidized by H_2O_2, the same hydrocarbons are generated as are exhaled *in vivo* [7], indicating that their origin in the organism might be related to similar reactions. In these tests ethane and pentane are formed during peroxidation of unsaturated fatty acids, whereas ethylene, propane and butane are released from hemoglobin by radical attack. Several reports dealing with such reactions on proteins and amino acids [8–11] encouraged us to develop a model system, that could imitate situations *in vivo* and release hydrocarbons from proteins and amino acids in the absences of unphysiologically high concentrations of H_2O_2.

Reactive oxygen species were generated by iron-catalysed autoxidation of ascorbate, a well known reaction that might occur *in vivo* [12]. Iron was used in minute amounts complexed with citrate, which is assumed to be a physiological iron chelator [13]. Glutathione is oxidized by autoxidizing ascorbate [14] and transfers hydrogen atoms to carbon-centered radicals [15]. The following experiments were therefore performed with and without addition of GSH to iron/ascorbate. The effect of inhibitors and the pH dependence of these model reactions were investigated in order to obtain information about the oxygen species involved in the release of volatile hydrocarbons from amino acids.

MATERIALS AND METHODS

Chemicals. GSH was from Boehringer (Mannheim, F.R.G.); superoxide dismutase from bovine erythrocytes (3200 units per mg protein), catalase from bovine liver (thymol-free, 17,500 units per mg protein), k-casein, bovine serum albumin (essentially fatty acid free), and all other chemicals of analytical grade were from the Sigma Chemical Co. (München, F.R.G.).

Incubation conditions. Reaction mixtures were incubated under hydrocarbon-free air in 12.5 mL headspace vials sealed with a rubber septum and containing 3 mL of Chelex-treated 0.1 M sodium phosphate buffer, pH 7.4. If not mentioned otherwise, final concentrations of added reagents were: ascorbate 0.5 mM; GSH 7.5 mM; $FeCl_3$ 5 μM; citrate 1 mM; amino acids 1 mM or proteins 10 mg/mL. Vials were shaken in the dark at 37° for 2 hr, and the headspace was subsequently analysed for hydrocarbons.

Analysis of hydrocarbons. After incubation 0.95 mL of the gas phase was removed with a gas-tight syringe and analysed for hydrocarbons by GLC according to Frank *et al.* [2]. A calibration mixture of hydrocarbons, obtained from Messer Griesheim (Frankfurt, F.R.G.), was used to estimate concentrations from peak heights. Amounts were calculated as nmol/L liquid phase. Each value (Tables 1 and 2) represents the mean of three different experiments.

RESULTS

Figure 1 demonstrates the specificity of ethylene, propane, and ethane generation from Met, Leu and Ile, respectively, when incubated in an iron/

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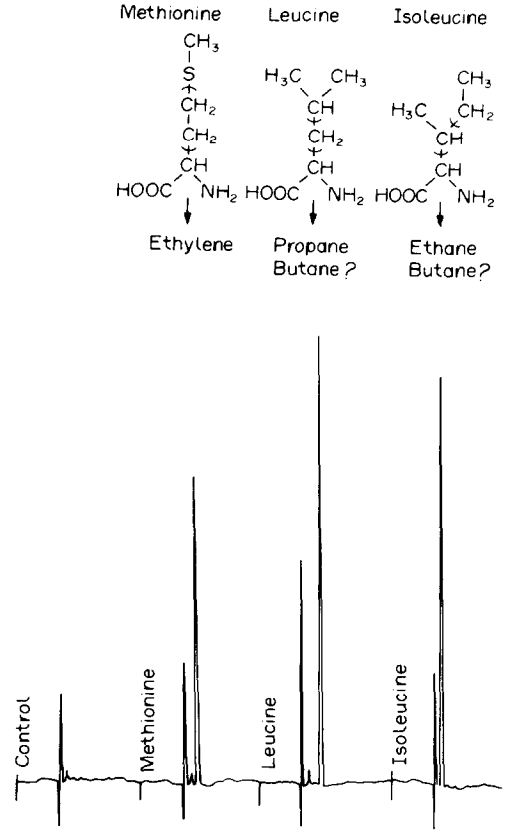

Fig. 1. Volatile hydrocarbons from amino acids.

ascorbate/GSH system. Other proteinic amino acids tested did not release these hydrocarbons. GSH, when added to an iron/ascorbate system, inhibited ethylene generation from Met in a concentration-dependent manner, presumably by scavenging reactive oxygen species and radicals derived from oxidized Met (Fig. 2, I). GSH, however, induced the release of propane from Leu and ethane from Ile. Time dependence studies showed a linear increase of propane and ethane during the first hour (Fig. 2, II), whereas the rate of ethylene generation increased after a lag phase in the presence or absence of GSH. Whereas the amount of hydrocarbons released was proportional to the concentrations of amino acids in the iron/ascorbate/GSH system (Fig. 2, III), Met inhibited its own ethylene generation in the iron/ascorbate system, probably by the release of thiyl radicals which scavenged other intermediate radicals (Fig. 2, III). The iron/ascorbate systems differ in their pH optima in the presence or absence of GSH (Fig. 2, IV). Ethylene showed a maximum at neutral pH in both systems, but yields of propane and ethane generation were highest at pH 6.2 in the presence of GSH.

Results of inhibition experiments are shown in Table 1. Catalase and hydroxyl radical scavengers like mannitol, dimethyl sulfoxide (Me_2SO), and thiourea efficiently reduced ethylene generation from Met in the iron/ascorbate system. Superoxide dismutase (SOD) was a relatively weak inhibitor, whereas BSA as a non-enzymatic protein showed

Table 1. Inhibition of hydrocarbon generation from Met, Leu and Ile (1 mmol/L each) in the presence of ascorbate (0.5 mmol/L) + Fe^{3+} (5 μmol/L) – citrate (1 mmol/L)

	Ethane nmol/L 2 hr	% Inhibition	Ethylene nmol/L 2 hr	% Inhibition	Propane nmol/L 2 hr	% Inhibition
+	0.0		392 ± 21		0.0	
+ mannitol 50 mmol/L	0.0		19.8 ± 0.8	95	0.0	
+ Me_2SO 50 mmol/L	0.0		4.0 ± 0.1	99	0.0	
+ Thiourea 50 mmol/L	0.0		2.1 ± 0.1	100	0.0	
+ BSA 50 mg/mL	0.0		202 ± 21	51	0.0	
+ SOD 166 μg/mL	0.0		184 ± 15	53	0.0	
+ Catalase 100 μg/mL	0.0		7.7 ± 0.2	98	0.0	
+ GSH 7.5 mmol/L	33.5 ± 1.3		11.1 ± 0.6		38.8 ± 1.6	
+ mannitol 50 mmol/L	16.7 ± 0.5	50	4.9 ± 0.2	56	20.3 ± 1.1	48
+ Me_2SO 50 mmol/L	14.5 ± 0.7	57	2.3 ± 0.1	79	14.2 ± 0.9	63
+ thiourea 50 mmol/L	6.4 ± 0.3	81	0.8 ± 0.0	93	5.4 ± 0.1	86
+ SOD 166 μg/mL	5.4 ± 0.2	84	0.6 ± 0.0	95	5.9 ± 0.1	85
+ catalase 100 μg/mL	10.1 ± 0.6	70	1.2 ± 0.1	89	10.0 ± 0.7	74

Incubation conditions as in Materials and Methods; N = 3; mean ± SD.

Table 2. Hydrocarbon generation from peptides and proteins in the presence of ascorbate 0.5 mmol/L + Fe^{3+} 5 μmol/L – citrate 1 mmol/L

	Ethane	Ethylene	Propane	Butane
	nmol/L			
+ Met 1 mmol/L	0.0	718 ± 14	0.0	0.0
+ Met-peptide 1 mmol/L	0.0	0.0	0.0	0.0
+ GSH 7.5 mmol/L + Leu 10 mmol/L	0.0	0.0	422.0 ± 16.0	0.0
+ Leu-peptide 10 mmol/L	0.0	0.0	167.0 ± 12.0	5.5 ± 0.3
+ BSA 10 mg/mL	0.0	0.0	0.0	0.0
+ GSH 7.5 mmol/L	14.8 ± 0.4	0.0	20.2 ± 0.5	2.8 ± 0.1
+ casein 10 mg/mL	0.0	0.0	0.0	0.0
+ GSH 7.5 mmol/L	7.9 ± 0.2	0.0	13.5 ± 0.3	7.8 ± 0.1

Incubation conditions as in Materials and Methods; N = 3; mean ± SD.
Met-peptide: *N*-formyl-L-methionyl-L-alanine.
Leu peptide: DL-alanyl-DL-leucyl-glycine.

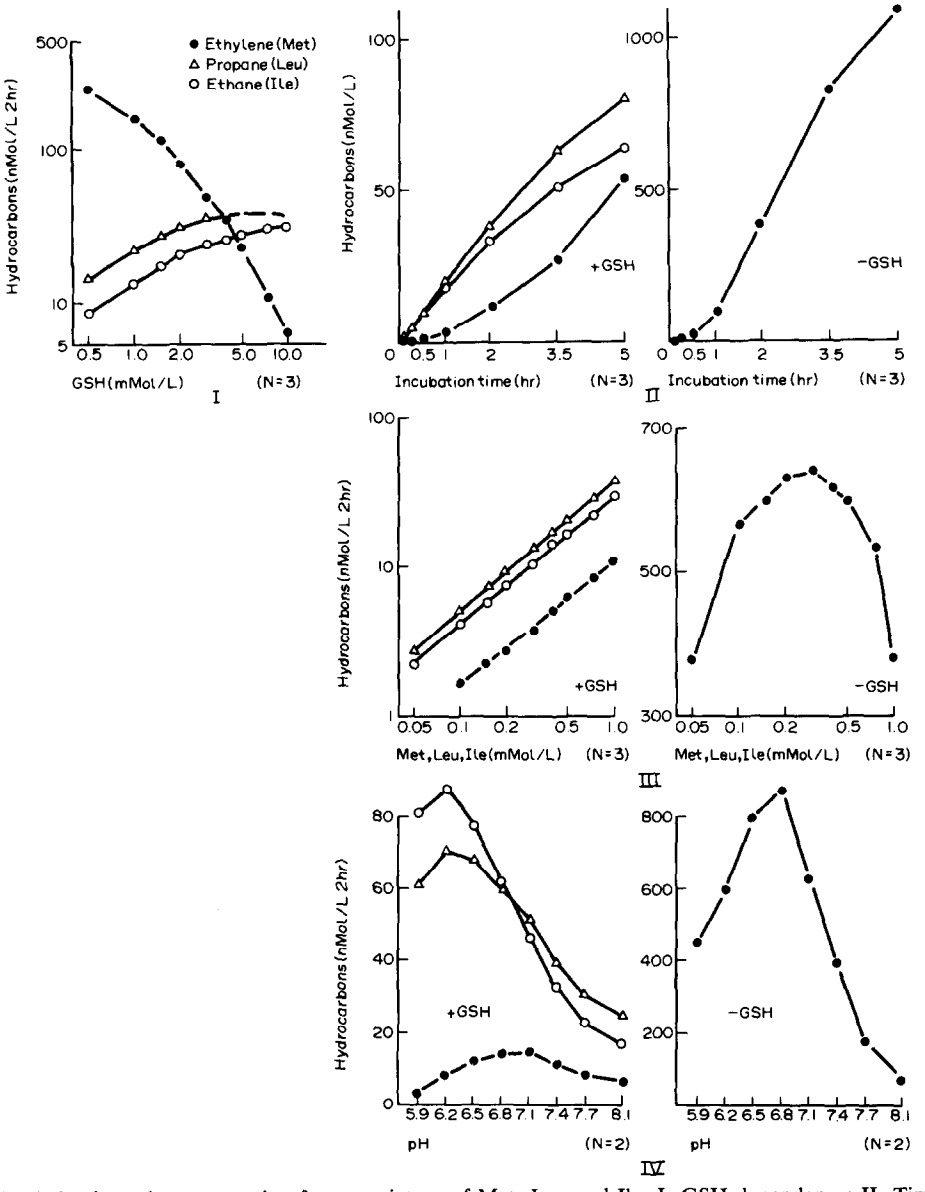

Fig. 2. Hydrocarbon generation from a mixture of Met, Leu and Ile: I. GSH dependence; II. Time dependence; III. amino acids concentration dependence; IV. pH dependence. Incubation conditions if not varied: 2 hr at 37°; phosphate buffer pH 7.4; Met, Leu, Ile 1 mmol/L; ascorbate 0.5 mmol/L; GSH 7.5 mmol/L; Fe^{3+} 5 μmol/L-citrate 1 mmol/L. Each point represents the mean of three different experiments.

only an unspecific inhibitory effect at a high concentration. In the iron/ascorbate/GSH system, however, SOD, thiourea and catalase were the most potent inhibitors, but mannitol and dimethylsulfoxide showed weaker potencies.

Expanding these findings to peptide-bound amino acids, we tested *N*-formyl-L-methionyl-L-alanine as a Met-peptide and DL-alanyl-DL-leucyl-glycine as a Leu-peptide. Peptide-bound Met did not yield any ethylene in the iron/ascorbate system, but peptide-bound Leu released small amounts of butane in addition to propane in the iron/ascorbate/GSH system (Table 2). Proteins like BSA or k-casein, which contain the amino acids tested, showed comparable results, but generated more butane compared to propane.

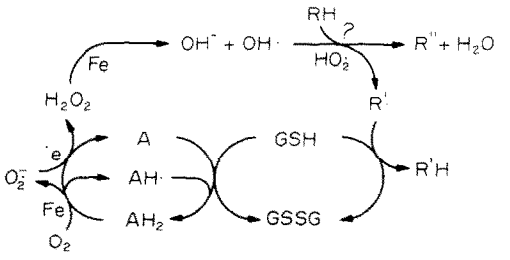

Fig. 3. Proposed mechanism for hydrocarbon release from Leu and Ile in the iron/ascorbate/glutathione system.

DISCUSSION

The mechanism of ethylene generation from Met or Met-like substances has been discussed for 20 years [16]. Hydroxyl radical, generally believed to be one of the most reactive oxygen species known, may play a major role in the reaction [17]. This radical is formed from superoxide and hydrogen peroxide by Haber–Weiss reaction or Fenton reactions [18]. Hydrogen peroxide production during iron-catalysed autoxidation of ascorbate is generally accepted, but there has been a long-standing controversy about whether superoxide is also generated [19, 20], although such evidence has been shown recently [21]. Our results of the inhibition of ethylene formation in the iron/ascorbate system are in accordance with superoxide generation as demonstrated by the specificity of SOD compared to the lack of specificity of BSA. Experiments with peptide-bound Met, which did not release ethylene, confirmed earlier results indicating a Strecker degradation to methional, i.e. an oxidative decarboxylation of α-amino acids, as a necessary intermediate of the ethylene generation [16]. This reaction may also explain the observed lag phase of the ethylene release whereas the following rapid rate increase may be due to intermediates which propagate a radical chain in addition to the oxygen radicals.

The formation of ethane and propane as a consequence of a radical attack on leucine and isoleucine has not yet been described. Radicals were identified after γ-irradiation of polycrystallized leucine and isoleucine. They originated from the scission of a C—H bond [22], but a release of alkanes was not determined. The ethane and propane generation was not possible in the Fe^{3+}–ascorbate system. The addition of GSH was essential for transmitting a hydrogen atom to the ethyl and propyl radicals. Figure 3 shows a scheme which combines the main reactions assumed to take place: the autoxidation of ascorbate and glutathione, catalysed by Fe; the reduction of oxidized ascorbate by glutathione; the OH-radical formation probably due to a Fenton-type reaction; and the transfer of a H-atom from GSH to the ethyl or propyl radical which was described in principle by Forni and Willson [15]. GSH has also been discussed controversially in relation to superoxide production during its autoxidation [23, 24], but now it seems to be proved [21] that O_2^- can be formed also by GSH. As indicated by the inhibition experiments presented here, the same oxygen species as in the iron/ascorbate system, being responsible for radical attack, seems to split the C—H bond of the γ-C atom of Leu and Ile, which is in accordance with radicals produced by γ-irradiation [22]. Peptide-bound Leu additionally yielded butane, indicating a radical attack at the β-C atom. In contrast to the ethylene formation from Met hydrocarbon, release from Leu and Ile seems not to be preceded by a Strecker degradation as indicated by the lack of a lag phase. In addition, the constant rates of the release of ethane and propane seem to exclude the participation of intermediate radicals.

The components of the iron/ascorbate/GSH system are ubiquitous in the body, indicating that similar reactions described here take place *in vivo* as experiments with normal rats exhaling the same alkanes could demonstrate [25]. The examination of the relevance of this system not only in regard to hydrocarbon exhalation but also to other aspects of oxygen radical attack on macromolecules could be important for evaluating the consequences originating from such a "steady-state" generation of reactive oxygen species. It is now possible, by measuring exhaled alkanes, to distinguish between peroxidation of unsaturated fatty acids, indicated by the production of ethane and pentane, and oxygen radical attack on amino acids, peptides and proteins, which split off ethane, ethylene, propane and butane but not pentane [26].

REFERENCES

1. Dillard CJ, Dumelin EE and Tappel AL, Effect of dietary vitamin E on expiration of pentane and ethane by the rat. *Lipids* **12**: 109–114, 1977.
2. Frank H, Hintze T, Bimboes D and Remmer H, Monitoring lipid peroxidation by breath analysis: endogenous hydrocarbons and their metabolic elimination. *Toxicol Appl Pharmacol* **56**: 337–344, 1980.
3. Filser JG, Bolt HM, Muliawan H and Kappus H, Quantitative evaluation of ethane and *n*-pentane as indicators of lipid peroxidation *in vivo*. *Arch Toxicol* **52**: 135–147, 1983.
4. Remmer H, Hintze T, Frank H and Müh-Zange M, Cytochrome P-450 oxidation of alkanes originating as scission products during lipid peroxidation. *Xenobiotica* **14**: 207–219, 1984.
5. Gelmont D, Stein RA and Mead JF, The bacterial origin of rat breath pentane. *Biochem Biophys Res Commun* **102**: 932–936, 1981.

6. Hintze T, Dissertation Tübingen, 1981 and Kessler W, Dissertation Tübingen, 1987.
7. Clemens MR, Einsele H, Frank H, Remmer H and Waller HD, Volatile hydrocarbons from hydrogen peroxide-induced lipid peroxidation of erythrocytes and their cell components. *Biochem Pharmacol* **32**: 3877–3878, 1983.
8. Levine RL, Oxidative modification of glutamine synthetase. *J Biol Chem* **258**: 11823–11827, 1983.
9. Wolff SP and Dean RT. Fragmentation of proteins by free radicals and its effect on their susceptibility to enzymic hydrolysis. *Biochem J* **234**: 399–403, 1986.
10. Gutteridge JMC, Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. *FEBS Lett* **128**: 343–346, 1981.
11. Zs-Nagy I and Floyd RA, Hydroxyl free radical reactions with amino acids and proteins studied by electron spin resonance spectroscopy and spin-trapping. *Biochim Biophys Acta* **790**: 238–250, 1984.
12. Roeser HP, The role of ascorbic acid in the turnover of storage iron. *Semin Hematol* **20**: 91–100, 1983.
13. Baker MS and Gebicki JM, The effect of pH on yields of hydroxyl radicals produced from superoxide by potential biological iron chelators. *Arch Biochem Biophys* **246**: 581–588, 1986.
14. Pirie A and van Heyningen R, Oxidation of glutathione in extracts of lens in the presence of ethylenediaminetetraacetate. *Nature* **173**: 873–874, 1954.
15. Forni LG and Willson RL, Electron and hydrogen atom transfer reactions: determination of free radical redox potentials by pulse radiolysis. *Methods Enzymol* **105**: 179–188, 1984.
16. Lieberman M and Kunishi AT, Ethylene production from methionine. *Biochem J* **97**: 449–459, 1965.
17. Beauchamp C and Fridovich T, A mechanism for the production of ethylene from methional. *J Biol Chem* **245**: 4641–4646, 1970.
18. Aust SD, Morehouse LA and Thomas CE, Role of metals in oxygen radical reactions. *J Free Radicals Biol Med* **1**: 3–25, 1985.
19. Puget K and Michelson AM, Iron containing superoxide dismutases from luminous bacteria. *Biochimie* **56**: 1255–1267, 1974.
20. Halliwell B and Foyer CH, Ascorbic acid, metal irons and the superoxide radical. *Biochem J* **155**: 697–700, 1976.
21. Rigo A, Scarpa M, Argese E, Ugo P and Viglino P, In: *Oxygen Radicals in Chemistry and Biology* (Eds. Bors W, Saran M and Tait D), pp. 171–176. Walter de Gruyter, Berlin, 1984.
22. Makino K and Riesz P, E.S.R. of spin-trapped radicals in γ-irradiated polycrystalline amino acids. Chromatographic separation of radicals. *Int J Radiat Biol* **41**: 615–624, 1982.
23. Misra HP, Generation of superoxide free radical during the autoxidation of thiols. *J Biol Chem* **249**: 2151–2155, 1974.
24. Thien M, Bucher JR and Aust SD, Thiol-dependent lipid peroxidation. *Biochem Biophys Res Commun* **107**: 279–285, 1982.
25. Remmer H, Kessler W, Einsele H, Hintze T, Diaz de Torango G, Garaibeh AM and Frank H, Ethanol promotes O_2-radicals attacking proteins, but not lipids. *Drug Metab Rev* **20**: 219–232, 1989