encing the intramolecular isotopic distribution in lipid components. The pyruvate internal isotopic arrangement is in turn a function of the isotopic distribution in its metabolic precursors. In plants, the cyclic nature of the Calvin cycle produces random incorporation of ^{14}C into photosynthetic carbohydrate in the long run (*12*), which implies that there will be no large isotopic differences in the carbon atoms of the hexoses which are the basic starting materials for metabolism. In animals, intramolecular isotopic distribution in carbohydrates would be a function of the relative contribution of de novo synthesis and assimilation of precursors from the diet; the further an animal is from plant carbon in the food chain, the more complex the factors determining the intramolecular isotopic arrangement of its pyruvate.

Techniques that allow for precise comparison of the isotopic composition of carbon atoms of lipid components which are known to derive from the methyl and carbonyl carbon atoms of acetyl CoA are not known at present. Their development would permit the direct demonstration of temperature-dependent intramolecular carbon isotopic heterogeneity in extant lipid components and would provide a method which might yield information as to the source and early thermal history of fossil lipids.

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References and Notes

1. Carbon isotopic composition is determined by converting the sample to CO_2 and comparing the $^{13}C^{12}C$ ratio of the sample with that of a standard by mass spectrometry. The results are reported in the notation

$$\delta^{13}C \text{ (per mil)} = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1\right] \times 10^3$$

For the work reported here, the standard is the Pee Dee belemnite (PDB) carbonate.
2. R. Park and S. Epstein, *Plant Physiol.* **36**, 133 (1961).
3. P. H. Abelson and T. C. Hoering, *Proc. Natl. Acad. Sci. U.S.A.* **47**, 623 (1961).
4. P. L. Parker, *Geochim. Cosmochim. Acta* **28**, 1155 (1964); B. N. Smith and S. Epstein, *Plant Physiol.* **46**, 738 (1970).
5. Two hypotheses have been advanced, one by Park and Epstein (*2*) and one by A. A. Ivlev, M. Ya. Koreleva, A. G. Kaloshin [*Dokl. Akad. Nauk SSSR* **217**, 224 (1974)]. The results of this study are not consistent with the main features of either hypothesis.
6. Most of the carbon atoms of the lipid fraction derive from carbon atoms 1, 2, 5, and 6 of the glucose molecule. If these atoms were sufficiently depleted in ^{13}C relative to carbon atoms 3 and 4 of glucose, the ^{13}C depletion of the lipid fraction could be explained without recourse to isotopic effects during metabolism.
7. E. Umbarger and B. D. Davis, in *The Bacteria*, I. C. Gunsalus and R. Y. Stanier, Eds. (Academic Press, New York, 1962), vol. 3, p. 241.
8. H. W. Doelle, *Bacterial Metabolism* (Academic Press, New York, ed. 2, 1975), p. 431.
9. L. J. Reed and R. M. Oliver, *Brookhaven Symp. Biol.* **21**, 397 (1969).
10. R. Breslow, *Chem. Ind.*, (1957), p. 893; M. F. Utter, in *The Enzymes*, P. D. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, New York, ed. 2, 1961), vol. 5, p. 322.
11. A full interpretation of the kinetic isotope effect data in terms of the rate-determining steps of the pyruvate decarboxylase reaction mechanism is in preparation (M. J. DeNiro and S. Epstein).
12. S. Aronoff and L. Vernon, *Arch. Biochem.* **28**, 424 (1950); M. Calvin, J. A. Bassham, A. A. Benson, V. H. Lynch, C. Ouellet, L. Schou, W. Stepka, N. E. Tolbert, *Symp. Soc. Exp. Biol.* **5**, 284 (1951).
13. H. L. Kornberg and J. R. Sadler, *Biochem. J.* **81**, 503 (1961).
14. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
15. H. Craig, *Geochim. Cosmochim. Acta* **3**, 53 (1953).
16. W. L. Roth and G. K. Rollefson, *J. Am. Chem. Soc.* **64**, 1707 (1942).
17. H. Sakai, J. W. Smith, I. R. Kaplan, C. Petrowski, *Geochem. J.* **10**, 85 (1976).
18. Supported by NSF grant DES71-00558 A03. This is contribution No. 2854, Division of Geological and Planetary Sciences, California Institute of Technology.

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A Bactericidal Effect for Human Lactoferrin

Abstract. Streptococcus mutans *and* Vibrio cholerae, *but not* Escherichia coli, *were killed by incubation with purified human apolactoferrin. Concentrations of lactoferrin below that necessary for total inhibition resulted in a marked reduction in viable colony-forming units. This bactericidal effect was contingent upon the metal-chelating properties of the lactoferrin molecule.*

Lactoferrin (LF), an iron-binding protein synthesized by neutrophils (*1*) and glandular epithelial cells (*2*), has been detected in most of the major secretions that bathe the human mucosal surfaces (*3*). Because it retards the growth of bacteria (*2, 4, 5*) and fungi (*6*) in vitro, LF has been suggested as a possible contributor to defense against local mucosal infections (*3*). The antimicrobial action of LF and its serum counterpart, transferrin (TF), has been attributed to the ability of these compounds to chelate iron, an action that makes this essential nutrient inaccessible to an invading microorganism (*7*). The ready reversibility of this antimicrobial effect, when an excess of iron is supplied to the nutritionally deprived organisms, suggests a simple bacteriostatic effect for LF. Our study, however, provides evidence that LF is capable of a direct bactericidal action on certain bacteria.

Purified LF was obtained as follows: (i) globulins were separated from a pool of human colostrum by ammonium sulfate precipitation, (ii) passed over CM (carboxymethyl) Sephadex C-50, (iii) subjected to ion exchange chromatography on A-25 (diethylaminoethyl) DEAE Sephadex, and (iv) filtered on Sephadex G-200 (*8*). The purity of the LF was assessed by immunoelectrophoresis against antiserum to normal human serum and antiserum to normal human colostrum and by disk-gel electrophoresis (*8*). Apo LF (iron-free) and saturated LF were prepared by dialysis against either 0.1*M* citric acid (*p*H 2.3) or saturated ferrous ammonium sulfate, respectively, then each was dialyzed against double-distilled water (*8*).

Three types of pathogens associated with exocrine secretions and mucosal surfaces were selected for this study: *Streptococcus mutans*, an etiologic agent of dental caries in humans and animals; *Vibrio cholerae*, the causative agent of Asiatic cholera; and *Escherichia coli*, an enteropathogen isolated from an infant diarrhea. *Streptococcus mutans* AHT (Bratthall serotype a) was grown under an atmosphere of 5 percent CO_2 in N_2 at 37°C for 18 hours in a partially defined (PD) medium containing 120 μM iron as $FeCl_3$. *Vibrio cholerae* was grown under conditions of constant shaking in 1.5 percent peptone broth at *p*H 8.0 and 37°C for 6 hours. *Escherichia coli* 0126 B16, also subjected to constant shaking, was cultured in PD-glucose medium at 37°C for 6 hours. Each culture was washed three times in sterile saline and resuspended to a concentration of approximately 2.0×10^9 colony-forming units (CFU) per milliliter.

In sterile cuvettes, 100 μl (2×10^8 CFU) of the various bacterial suspensions were incubated with 200 μl of various concentrations of LF (1.0 to 2000 μg) for 1 hour at 37°C. After incubation, 2.7 ml of the appropriate medium was added to the reaction mixture, and growth at 37°C was monitored spectrophotometrically at 660 nm (Fig. 1). The growth of *S. mutans* was totally inhibited by prior incubation with 50 or more micrograms of LF per 300 μl ($\geq 2\ \mu M$) of reaction mixture (Fig. 1A). Furthermore, with concentrations less than 50 μg, there was a dose-dependent delay in the onset of detectable growth. Similarly, *V. cholerae* was totally inhibited by LF concentrations of or in excess of 100 μg and, at lesser concentrations, showed a delay in the onset of exponential growth proportional to the LF concentrations (Fig. 1B). In contrast, the growth of *E. coli* was not inhibited by concentrations of apo LF as high as 2000 μg per reaction mixture (80 μM). Saturated LF, in concentrations of 2000 μg, did not alter the

Table 1. The effect of various concentrations of lactoferrin on *Streptococcus mutans* AHT, *Vibrio cholerae* 569B, and *Escherichia coli* 0126. The data are expressed as colony-forming units per reaction volume; *S. mutans* was grown on Mitis-Salivarius agar, *V. cholerae* on 1.5 percent peptone agar (*p*H 8.0), and *E. coli* on 1 percent BHI agar.

Species	Saturated lactoferrin*	Concentration of apolactoferrin (micrograms per 300 μl of reaction mixture)						
		1.0	10.0	20.0	50.0	100.0	1000.0	Control†
S. mutans AHT	7.0×10^7	6.5×10^7	5.0×10^6	1.9×10^4	NG‡	NG	NG	7.2×10^7
V. cholerae 569B	8.8×10^7	8.2×10^7	7.8×10^6	1.9×10^6	9.2×10^4	NG	NG	8.5×10^7
E. coli 0126	9.2×10^7	8.1×10^7	8.2×10^7	8.0×10^7	7.2×10^7	7.2×10^7	6.5×10^7	8.5×10^7

*Tested at a concentration of 1000 μg. †Bacteria incubated in saline. ‡No growth.

growth of any of the bacterial species tested.

The total inhibition of growth of *V. cholerae* and *S. mutans*, when subcultured in iron-rich medium after prior incubation with LF, suggested that LF was bactericidal for these organisms. The delay in onset of detectable growth, apparent with lesser concentrations of LF, could likewise be explained by a reduction in viable CFU. To test this assumption, a further series of experiments was performed. Portions of the various reaction mixtures were incubated with LF for 1 hour and then serially diluted and plated in duplicate.

Streptococcus mutans was cultured on Mitis-Salivarius agar under 5 percent CO_2 in N_2 for 48 hours at 37°C. *Vibrio cholerae* was plated on 1.5 percent peptone agar at *p*H 8.0 and *E. coli* on beef heart infusion (BHI) agar; both were grown at 37°C for 18 hours. The values obtained from this bactericidal assay (Table 1) reflected the growth dynamics observed in the spectrophotometric studies. Total inhibition of *S. mutans* growth was obtained by LF concentrations of 50 or more micrograms, and a significant reduction in CFU was observed with as little as 10 μg of LF in the reaction volume. The cholera organism showed marked reduction in CFU with 20 μg of LF and total inhibition with 100 or more micrograms of LF. A significant reduction in CFU was not observed with *E. coli* even with LF concentrations as high as 1000 μg (Table 1). Again, saturation of LF with iron eliminated the bactericidal activity because the number of CFU obtained when the organism was incubated with concentrations of saturated LF in excess of that necessary for total inhibition by apo LF was equivalent to control numbers (Table 1).

These observations suggest that certain bacteria are directly affected by the chelating properties of LF, perhaps at the bacterial cell surface. To determine whether LF was binding to the cell surface, we conducted a separate series of experiments using immunofluorescent microscopy (*9*). Both *S. mutans* and *V. cholerae* exhibited positive fluorescence with apo LF but not with saturated LF. When incubated with saturated LF, as well as with apo LF, *E. coli* exhibited positive fluorescence. Control smears without LF showed negative fluorescence for all microorganisms.

The inhibition of bacterial growth observed in these experiments was probably not due to LF binding the available iron in the media. The amount (50 μg) of apo LF that inhibited *S. mutans* was the equivalent to the 0.2 μ*M* in the 3.0 ml of culture medium that contained 120 μ*M* iron. This is considerably less than the minimum 60 μ*M* LF that would be necessary to bind all available iron. The peptone broth used to grow the *V. cholerae* probably contained 4.0 to 7.0 μ*M* iron (*10*), which would require 2.0 to 3.5 μ*M* apo LF for complete binding. Total inhibition, however, was observed with as little as 0.4 μ*M* LF. In addition, the reduction in CFU observed (Table 1) can only be explained by a bactericidal effect.

The ability to synthesize iron chelators (enterochelins) has been suggested as a significant virulence factor for several enteropathogenic strains of *E. coli* as well as for certain other pathogens (*4, 11, 12*). Based on the assumption that the chelating properties of LF act nutritionally to deprive bacteria of essential iron, results of these investigations have suggested that synthesis of enterochelins allows the bacterium to utilize LF- or TF-bound iron. The resistance of *E. coli* 0126 to the bactericidal action of LF in the present study may also be caused by the production of iron chelators. The positive fluorescence observed with *E. coli* 0126, but not with *S. mutans* or *V. cholerae* when incubated with saturated LF, may be caused by a cell-associated enterochelin. If an enterochelin or enterochelin-like substance is responsible for the resistance of this bacterium to the bactericidal effect of apo LF, then its action is probably more subtle than simple chelation of LF-bound iron. It is interesting that one group of investigators has studied a strain of *E. coli* for which LF is bacteriostatic at low concentrations, but may be bactericidal at high concentrations (*12*). It may be that LF in sufficient amounts can overcome the resistance afforded by enterochelin synthesis. Further comparative studies of the effects of LF on organisms known to synthesize

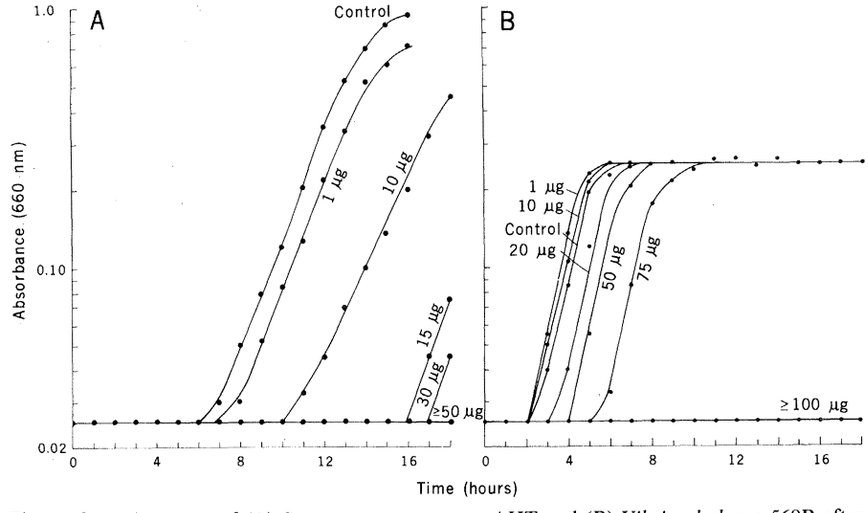

Fig. 1. Growth curves of (A) *Streptococcus mutans* AHT and (B) *Vibrio cholerae* 569B after incubation of approximately 2×10^8 CFU with varying concentrations of apo LF (1.0 μg to 1000 μg per 300 μl of reaction mixture) for 1 hour at 37°C. Growth curves with saturated LF were no different from the control. The treated bacteria were grown in 2.7 ml of either PD-glucose medium (*S. mutans*) or 1.5 percent peptone broth at *p*H 8.0 (*V. cholerae*). The optical density was monitored (660 nm) with a Beckman DB-G spectrophotometer equipped with a water-jacketed multiple-sample turret maintained at 37°C and recorded on a Beckman recorder.

iron-binding molecules, as well as those that do not, may provide insight into both the bactericidal mechanism of, and the bacterial resistance to, the action of LF.

Both the spectrophotometric and bactericidal assays indicate that on certain microorganisms, LF is capable of exerting a direct bactericidal effect that is contingent upon its chelating properties. This would suggest that LF may be capable of a similar mechanism in mucosal secretions and in neutrophils (*13*). Both the Gram-negative *V. cholerae* and the Gram-positive *S. mutans* were highly susceptible to the bactericidal action of LF at concentrations within the physiological range of many secretions and with numbers of bacteria normally encountered in situ.

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References and Notes

1. M. Baggiolini, D. deDuve, P. L. Masson, J. F. Heremans, *J. Exp. Med.* **131**, 559 (1970); I. Green, C. H. Kirkpatrick, D. C. Dale, *Proc. Soc. Exp. Biol. Med.* **137**, 1311 (1971); M. S. Leffell and J. K. Spitznagel, *Infect. Immun.* **6**, 761 (1972); J. K. Spitznagel, F. G. Daldorf, M. S. Leffell, J. D. Folds, I. R. Welsh, M. H. Cooney, L. E. Martin, *Lab. Invest.* **30**, 774 (1974).
2. P. L. Masson, J. F. Heremans, J. J. Prignot, G. Wanters, *Thorax* **21**, 538 (1966).
3. P. L. Masson, J. F. Heremans, C. Dive, *Clin. Chim. Acta* **14**, 735 (1966).
4. J. J. Bullen, H. J. Rogers, L. Leigh, *Br. Med. J.* **1**, 69 (1972).
5. B. Reiter, J. H. Brock, E. D. Steel, *Immunology* **28**, 83 (1975); J. D. Oram and B. Reiter, *Biochim. Biophys. Acta* **170**, 351 (1968); B. Reiter and J. D. Oram, *Nature (London)* **216**, 328 (1968).
6. C. H. Kirkpatrick, I. Green, R. R. Rich, A. L. Schade, *J. Infect. Dis.* **124**, 539 (1971).
7. E. D. Weinberg, *Science* **184**, 952 (1974); I. Kochan, *Curr. Top. Microbiol. Immunol.* **60**, 1 (1973).
8. M. F. Cole, R. R. Arnold, J. Mestecky, S. Prince, R. Kulhavy, J. R. McGhee, *Microbial Aspects of Dental Caries* (Information Retrieval, Washington, D.C., 1976), vol. 2, p. 359.
9. Heat-fixed smears of washed bacteria were allowed to incubate for 45 minutes at room temperature with 0.1 percent concentrations of either apo LF or saturated LF. After washing, the smears were incubated with rabbit antiserum to human lactoferrin for 45 minutes, washed, and incubated with goat antiserum to rabbit IgG labeled with rhodamine isothiocyanate (Hyland). The washed smears were observed with a Leitz fluorescence microscope and vertical Ploem illumination.
10. E. S. Kempner, *Appl. Microbiol.* **15**, 1525 (1967).
11. H. J. Rogers, *Infect. Immun.* **7**, 445 (1973); *Immunology* **30**, 425 (1976); J. J. Bullen and H. J. Rogers, *Nature (London)* **224**, 380 (1969); G. A. Snow, *Bacteriol. Rev.* **34**, 99 (1970); T. D. Wilkins and C. E. Lankford, *J. Infect. Dis.* **121**, 129 (1970); I. Kochan, N. R. Pellis, C. A. Golden, *Infect. Immun.* **3**, 553 (1971).
12. J. G. Bishop, F. L. Schanbacher, L. C. Ferguson, K. L. Smith, *Infect. Immun.* **14**, 911 (1976).
13. M. S. Leffell and J. K. Spitznagel, *ibid.* **12**, 813 (1975).
14. This work was supported by NIH grants DE 04217-02 and DE-02670-10, and contract DE-52456-02. We thank S. Prince and R. Kulhavy for technical assistance and J. Morris for typing this manuscript.

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Fragile Sites on Human Chromosomes: Demonstration of Their Dependence on the Type of Tissue Culture Medium

Abstract. *The observation of heritable fragile sites on human chromosomes prepared from lymphocyte cultures has been shown to depend on the type of tissue culture medium in which the lymphocytes are grown. The sites are observed at a much greater frequency when medium 199 is used than when RPMI 1640, Ham's F10, Eagle's (basal), and CMRL 1969 are used. One site on the X chromosome is of clinical significance in that it is a marker for X-linked mental retardation.*

Heritable fragile sites have been described on a number of human chromosomes, including numbers 2, 12, 16, 17, X, and unidentified C-group chromosomes (*1, 2*). When the autosomes are involved these sites are apparently of no clinical significance, but the site on the X is associated with some forms of X-linked mental retardation (*3*).

I recently attempted to reexamine a fragile site on chromosome 2 which had been identified and shown to be familial some years before. Initial studies were puzzling because the fragile site appeared to have vanished. Investigation of this led to the finding that the frequency of lesions at the fragile site depended on the type of tissue culture medium in which the lymphocytes used to produce the chromosomes were cultured. This observation was extended to and confirmed for fragile sites on the X, 10, and 20 (Fig. 1).

Chromosome preparations from lymphocyte cultures grown in parallel in several different commercially available tissue culture media were scored for the presence or absence of a lesion at the fragile site on a number of occasions. The results are shown in Table 1. The frequency of lesions at the fragile site was always much greater when medium 199 was used than with any of the other media. This difference was not so pronounced when the frequency of the lesions was low. When a medium other than 199 was used the frequency of these sites was so low that they would have escaped detection by routine clinical chromosome analysis.

The reasons for these differences in frequency of breakage at the fragile sites according to the type of culture medium are unknown. They may represent either a nonspecific phenomenon related to factors such as *p*H or osmolarity, or be due to a specific chromosome breaking agent present at higher concentrations in medium 199 than the other media used or only present in 199. Delineation of the mechanisms responsible for this effect would be of considerable use.

The findings reported here are of importance in the study of X-linked mental

Table 1. Frequency of observation of fragile sites according to the type of tissue culture medium used. Tissue culture media used were purchased at single strength (except for Eagle's basal medium which was purchased as a × 10 concentrate) from Commonwealth Serum Laboratories, Melbourne, Australia. Lymphocyte cultures consisting of 4 ml of medium, 1 ml of fetal bovine serum, 0.1 ml of phytohemagglutinin, and 0.2 ml of venous blood were harvested according to standard methods after 72 hours of incubation; colchicine was applied for 2 hours and the hypotonic solution used was 0.075*M* KCl. A cell was considered to display evidence of a fragile site if one or both chromatids were broken at the site, if there was chromosome material either in addition or missing which corresponded to the parts of the chromosome on either side of the fragile site, or if a triradial figure was present. Results are expressed as number of cells showing a lesion at the fragile site over the total number of cells examined.

Subject No.	Fragile site	Date studied	Tissue culture medium				
			199	RPMI 1640	Ham's F10	Eagle's (basal)	CMRL 1969
1	2q1	September 1972	23/50				
		May 1976	34/55	3/55			
		June 1976	25/50	0/50	3/50	3/50	2/50
		September 1976	34/50		0/50		
2	10q23	October 1973	36/50				
		May 1976	22/55	1/55			
		June 1976	7/50	0/50	1/50	1/50	0/50
		September 1976	23/50		0/50		
3	20p11	November 1973	33/50				
		May 1976	13/50	5/50	3/50	2/50	
		September 1976	24/50	1/50	0/50	2/50	
4*	20p11	September 1976	22/50		0/50	2/50	
5†	Xq27 or 8	May 1976	8/50	5/50		0/50	
6‡	Xq27 or 8	May 1976	4/50	0/50	0/50	0/50	
		June 1976	5/50	0/50	0/50	0/50	

*Sibling of subject No. 3. †Male. ‡Mother of subject No. 5.