often replaces and takes the form of the decomposing body and forms concretions retaining much of the external morphology of the organism. A recent example is the occurrence of alewife concretions described by Sondheimer *et al.* (*12*) from calcium-rich Lake Onondaga. The x-ray diffraction pattern of one of these concretions is shown in Fig. 2 and is very similar to that of the material formed in the laboratory. The chemical composition of the Onondaga concretion (which consists mainly of calcium palmitate) is almost identical (in C:H:Ca+Mg ratio) to herring adipoceres described by Wells and Erickson (*13*) which had formed in seawater off the coast of Alaska.

Various mechanisms for the breakdown of fatty acids as processes involved in the formation of petroleum have been suggested by many workers (*14*). If such breakdown actually occurs, then an original adipocere concretion may eventually become a $CaCO_3$ concretion once the hydrocarbon portion is removed. Excellent preservation of fossils within $CaCO_3$ concretions is believed to be due to very rapid syngenetic formation of the concretion on or near the sea floor (*1*). If some concretions originally consisted of adipocere, such a rapid fossilization mechanism is reasonable in the light of the present laboratory experiments and the finding of adipoceres only tens of years old (*11*). The present experiments also demonstrate that even in the absence of fatty acid, abundant alkalinity is available from the decomposition of fish to precipitate calcium as $CaCO_3$ during the early stages of decay.

The conversion of adipocere to $CaCO_3$ because of a considerable density increase, would necessitate a shrinkage of the concretion. Although this effect should not greatly affect an enclosed skeleton, it could destroy external morphology. The finding of $CaCO_3$ replacements preserving external features, as described by Weeks (*1*), would necessitate a virtually isotropic shrinkage or later infilling by $CaCO_3$ from ground water, if the concretions were derived from original adipocere.

One possible test of an original adipocere origin for $CaCO_3$ concretions is the measurement of the C^{13}/C^{12} ratio of the carbonate. Adipocere and lipid carbon are highly depleted in C^{13} relative to normal marine calcium carbonate (*12*). During the breakdown of adipocere it is probable that the remaining calcium will form $CaCO_3$ from isotopically light carbon. Thus $CaCO_3$ concretions containing well-preserved fossils, which show a very low C^{13}/C^{12} ratio in the carbonate, may have formed originally as adipocere which was later converted to $CaCO_3$. Nonfossiliferous concretions very low in C^{13}, such as some of those described by Hodgson (*15*), may have formed in a similar manner.

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References and Notes

1. W. H. Twenhofel, *Treatise on Sedimentation* (Dover, New York, 1965), p. 714; L. G. Weeks, *Bull. Geol. Soc. Amer.* **68**, 95 (1957).
2. A good example is shown by the table in K. O. Emery, *The Sea off Southern California* (Wiley, New York, 1960), p. 276.
3. H. R. Krause, *Arch. Hydrobiol.* **24**, 297 (1959).
4. F. E. Hecht, *Senckenberg. Naturforsch. Gesellschaft* **15**, 165 (1933).
5. K. V. Thimann, *The Life of Bacteria* (Macmillan, New York, 1963).
6. No special precautions against aeration were taken during withdrawal of samples for chemical analysis or during measurement of *p*H. The presence of considerable dissolved sulfide (from breakdown of sulfur-containing organic compounds) as measured with a Ag_2S electrode was found in six out of seven runs after 120 to 170 days of rotting. This indicates the ability to reattain anaerobic conditions even after repeated opening of the jars and exposure to air. Also, aeration and decrease in volume due to sample withdrawal brought about no qualitative difference in chemical changes with time between runs sampled very infrequently ($<$ 10 percent volume decrease) and those sampled frequently ($>$ 50 percent volume decrease).
7. Chemical analysis of part of the material remaining in suspension after treatment with H_2O_2, which could be centrifuged down after 1 hour at 2000 rev/min, showed Ca/Mg $\sim$ 4 for one sample that had an x-ray pattern matching Ca fatty acid salts. Another sample showing an x-ray pattern for Ca salts had Ca/Mg $\sim$ 6. This sample was collected by settling in water for about 1 month.
8. E. H. Gruger, R. W. Nelson, M. E. Stansby, *J. Amer. Oil Chem. Soc.* **41**, 662 (1964).
9. The solubility products for the free fatty acids are based on the data of L. M. John and J. W. McBain, *J. Amer. Oil Chem. Soc.* **40** (1948). These values are lower than those of A. W. Ralston and C. W. Hoerr [*J. Org. Chem.* **7**, 546 (1942)] so that equilibrium values of P_{CO_2} may be even higher. Solubility product constants for calcium soap are taken from the precise work of R. R. Irani and C. F. Callis [*J. Phys. Chem.* **64**, 1741 (1960)].
10. R. M. Garrels and C. L. Christ, *Solutions, Minerals, and Equilibria* (Harper and Row, New York, 1965).
11. For an excellent discussion and summary of references on adipocere see W. Bergmann, in *Organic Geochemistry*, I. A. Breger, Ed. (Pergamon, New York, 1963), p. 517.
12. E. Sondheimer, W. A. Dence, L. R. Mattick, S. R. Silverman, *Science* **152**, 221 (1966).
13. R. C. Wells and E. T. Erickson, *J. Amer. Chem. Soc.* **55**, 338 (1933).
14. I. A. Breger, *Geochim. Cosmochim. Acta* **19**, 297 (1960); J. E. Cooper and E. E. Bray, *ibid.* **27**, 1113 (1963); W. D. Rosenfeld, *Arch. Biochem.* **16**, 263 (1948); S. R. Silverman, in *Isotopic and Cosmic Chemistry*, H. Craig, S. L. Miller, G. J. Wasserburg, Eds. (North-Holland, Amsterdam, 1964), p. 92.
15. W. A. Hodgson, *Geochim. Cosmochim. Acta* **30**, 1223 (1966).
16. Supported by grant 1937-A2 of the Petroleum Research Fund, American Chemical Society. Technical assistance was provided by Mrs. C. Thomlinson; concretions from Lake Onondaga were provided by E. Sondheimer.

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Dense-Gas Chromatography of Nonvolatile Substances of High Molecular Weight

Abstract. *Working at pressures of up to 2000 atmospheres, more than ten times higher than in previous gas chromatography, we used the solvent power of dense gases to enable migration of chromatographic substances of molecular weights as high as 400,000. Carotenoids, corticol steroids, sterols, nucleosides, amino acids, carbohydrates, and several polymers have been caused to migrate, separated, and detected in NH_3 and CO_2 carrier gases at temperatures of 140° and 40°C, just above the respective critical points. Previously such compounds either defied separation by gas chromatography or had to be chromatographed as their more volatile derivatives.*

Gas chromatography has been spectacularly successful for high-resolution, high-speed separation. Its range has been strictly limited, however, by the requirement that all substances must be reasonably volatile in order to achieve substantial partitioning with the gas phase; thus excluded are many challenging separations involving biochemical, inorganic, and macromolecular substances (*1*). This limitation can be moderated or even eliminated by use of dense-gas chromatography.

Dense gases promise to constitute an especially versatile class of mobile phases for chromatography; depending on the degree of compression and on the temperature relative to the critical point, their properties can range continuously between the extremes of ordinary liquids and ideal gases. Since liquids are approximately 1000 times denser than gases at atmospheric pressure, compression of gases to roughly 1000 atm yields fluids whose properties are generally liquid-like. Intermolecular forces play a dominant role and give the fluid a solvent power resembling that of a true liquid. If the proper gas is chosen, considerable solvent power may remain even when density (and pressure) is lowered from the level at which the gas closely simulates liquids. Thus one has considerable latitude in choosing pressure and temperature at which the gas will exhibit favorable solvent effects while still largely retaining the favorable viscous and diffusive characteristics that are fundamentally responsible for the advantages of gas chromatography. Moreover, one may

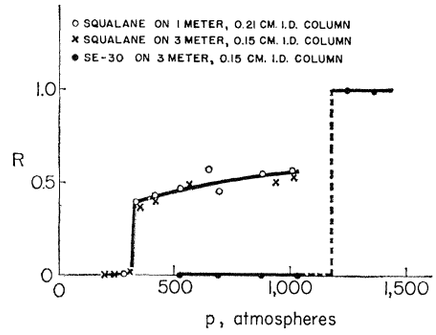

Fig. 1. Relative migration rate (R) versus pressure of CO_2 for squalane and silicone gum rubber (*SE-30*) in columns of 20 percent Apiezon-L on Chromosorb-W at 34°C.

choose low temperatures so as to avoid the decomposition of molecules having low thermal stability.

The fact that dense gases have solvent characteristics was first shown in 1879 (*2*). Until recently the chief practical consequence of this phenomenon was the dissolution, and subsequent unwanted deposition, of silica in steam turbines (*3*). The most thoroughly studied systems from the viewpoint of physical chemistry involve low-molecular-weight solids (such as CO_2) in gases such as helium, air, and methane at moderate pressures (about 100 atm) (*4*, *5*). The applicability to gas chromatography was first demonstrated by migration of two porphyrins in freon gases at 140 atm (*6*); later the general theoretical advantages of dense-gas chromatography were discussed by one of us (*7*). More recently Sie *et al.* have used pressures of up to 80 atm to enhance the migration of hydrocarbons (*8*). Although gas-chromatography equipment suitable for operation at up to 2000 atm has been developed and used in our laboratory, it has not been

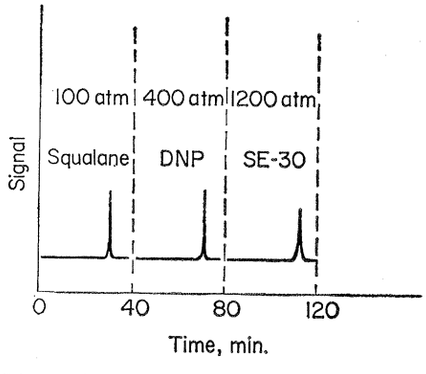

Fig. 2. Stepwise pressure programming of squalane, dinonyl phthalate (*DNP*), and silicone gum rubber (*SE-30*) in CO_2 at 40°C.

focused hitherto on such study of the solvent properties of dense gases (*9*).

The experimental apparatus was a modification of reported instrumentation (*9*). The CO_2 system was based on an Aminco (46-14021) air-driven diaphragm compressor capable of pressures of up to 2000 atm. For our work the compressor was heated to 40°C or higher in order to exceed the critical temperature of CO_2—31.1°C. A Whitey (LP 10) motor-driven diaphragm pump produced liquid-ammonia pressures of up to 340 atm; the liquid ammonia was then heated to 140°C to yield the dense gas (T_c for NH_3 is 132.3°C).

The injection system was similar to that reported, except that the reservoir volume was increased to approximately 5 cm^3. The substance under investigation was introduced into the reservoir prior to application of the pressure which was then maintained for 30 minutes before the headspace was sampled by flushing with carrier gas into the column inlet. For solubility studies, the column consisted of a short length of empty tubing, 0.05 cm in internal diameter. Conventional packed columns (internal diameter, 0.21 cm) were used for chromatographic separations. For maintenance of volatility the entire column was kept at high pressure by means of a valve at the column outlet. Beyond the valve there was a tendency for high-molecular-weight materials to become less soluble in the carrier gas and thus to condense to either produce a fog or be deposited on the wall before reaching the detector. These effects did not interfere with transport to the detector when the size of the sample was kept minimal and the line to the detector was heated. Detection was by a flame-ionization unit. Under the same conditions of flow and temperature, the detector signal was used as a measure of dense-gas solubility, and thus of the tendency to promote migration chromatographically.

In Fig. 1 we show migration-pressure curves for squalane and silicone gum rubber in CO_2, using Apiezon-L as stationary phase (Apiezon-L will itself migrate at about 1500 atm). The curves rise rather abruptly, a result expected exclusively for species of high molecular weight, in which many solvent molecules would be required to form the volatile "cluster." Studies with small molecules show less rapid variations, adequately described by second or third virial coefficients (*4*). It is significant that in some instances pressures must indeed be in the vicinity of 1000 atm for migration to be effected.

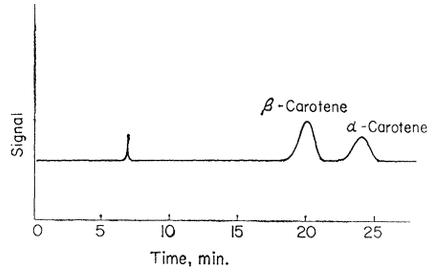

Fig. 3. Separation of α- and β-carotenes in CO_2 at 500 atm and 40°C on a 15-percent Ucon column.

The high sensitivity to pressure, shown by these migration rates, suggests that pressure may be used to control migration. It appears, in fact, that a major advantage of dense-gas chromatography, compared to conventional gas or liquid chromatography, is that a simple mechanical parameter, pressure, which is subject to precise and yet rapid variation, has enormous influence on solvent power and thus on velocity of migration. A programmed pressure system may be imagined, analogous to the successful gradient-elution methods in liquid chromatography or to programmed temperature in gas chromatography. In Fig. 2 we demonstrate the use of a sequence of pressure increments to attain separation; pressure was increased in steps to 100, 400, and 1200 atm; 30 minutes after each pressure increment, vapor from the reservoir was sampled. The last component to migrate was a silicone gum rubber having a molecular weight of 400,000. This rudimentary separation did not require a sorptive column to enhance resolution; it shows, in fact, that dense gases can even be effective as solvents for single-stage extractions.

Our preliminary experiments with CO_2 and NH_3 confirm that dense gases exhibit solvent properties that are analogous to those of liquids and that generally relate to molecular structure (*10*). Dense gaseous NH_3, like the liquid form of NH_3, shows solvent characteristics approximately intermediate between those of water and of an aliphatic alcohol of low molecular weight, except for its low acidity (*11*); its ability to form hydrogen bonds favors the solution of compounds containing hydroxylic, amino, and related groups. Our experiments show that amines, amino acids, di- and tripeptides, and mono- and disaccharides migrate readily (judged by detector response,

as we have mentioned) in gaseous NH_3. [Some free sugars probably react to form the corresponding amino sugars (*12*).]

Other more complex biochemicals, such as proteins and higher polysaccharides, did not migrate. Glucose migrates rapidly; galactose, only slowly—a result much like that in liquid NH_3. Nucleosides such as adenosine and guanosine migrate readily, but the corresponding purines (adenine and guanine) give no indication of chromatographic movement. It is generally accepted that the notorious insolubility of these purines in liquids is due to strong intermolecular forces, mainly hydrogen bonding, between solute molecules (*13*); this idea is again confirmed by our observation that caffeine (1,3,7-trimethylxanthine) migrates in dense gaseous NH_3 but xanthine does not. In addition to these examples, high-molecular-weight polyethylene glycols, such as the gas-chromatography stationary phases Carbowax-4000 and Carbowax-20M, migrate readily in dense NH_3 gas.

Solution in liquid CO_2 is believed to involve primarily dispersion, quadrupole and acid-base interactions (*14*). The CO_2 molecule can form an acceptor-donor complex with the highly polarizable π-electrons of multiple bonds. The order of solubility is roughly the order of unsaturation. Thus we observe that the terpenes, corticol steroids, and sterols migrate in dense CO_2 gas with progressively less solubility; the highly conjugated carotenoids migrate readily at 170 atm, while the above steroids barely migrate at 1300 atm. Amino acids do not migrate at all.

The necessity for careful choice of the gaseous solvent to be used in dense gas chromatography is illustrated by the migration of nucleosides and related compounds in NH_3 and CO_2. We find, as expected, that NH_3 is the better of the two solvents for carbohydrates, although CO_2 provides some migration. Conversely, CO_2 is the better for the difficult purines, causing a slight but promising migration. The nucleosides migrate slightly in CO_2 just like their constituent carbohydrate and purine fragments. As we have mentioned, NH_3 readily promotes migration of the nucleosides.

Such examples stress the unique migrational characteristics of dense gases, especially for biomolecules. Migration has been emphasized because it is an essential prerequisite to separation. The fact that useful separations of moderately large biomolecules can be achieved has been demonstrated by the direct separation in CO_2 at 40°C of ergosterol, cholesterol, and lanosterol; of squalene and squalane; and of α- and β-carotene. In Fig. 3 we show the last separation on a 1.5-m, 15-percent Ucon column at 500 atm (Ucon begins to come off at about 700 atm). Because of the instability of carotenoids, this separation is impractical by ordinary gas chromatography. With continued improvement of system parameters and further inquiry into the nature of dense-gas solubility and transport phenomena, dense-gas chromatography will probably be able to effect increasingly difficult separations.

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References and Notes

1. E. Heftmann, Ed., *Chromatography* (Reinhold, New York, ed. 2, 1967).
2. J. B. Hannay and J. Hogarth, *Proc. Roy. Soc. London* **29**, 324 (1879); **30**, 178 (1880).
3. R. S. Bradley, Ed., *High Pressure Physics and Chemistry* (Academic Press, New York, 1963), vol. 1, p. 4.
4. J. S. Rowlinson and M. J. Richardson, in *Advances in Chemical Physics*, I. Prigogine, Ed. (Interscience, New York, 1959), vol. 2, p. 85.
5. H. S. Booth and R. M. Bidwell, *Chem. Rev.* **44**, 477 (1949).
6. E. Klesper, A. H. Corwin, D. A. Turner, *J. Org. Chem.* **27**, 700 (1962).
7. J. C. Giddings, in *Gas Chromatography 1964*, A. Goldup, Ed. (Elsevier, Amsterdam, 1965); *Separation Sci.* **1**, 73 (1966).
8. S. T. Sie, W. van Beersum, G. W. A. Rijnders, *Separation Sci.* **1**, 459 (1966); S. T. Sie and G. W. A. Rijnders, *Anal. Chim. Acta* **38**, 31 (1961).
9. M. N. Myers and J. C. Giddings, *Anal. Chem.* **37**, 1453 (1965); *Separation Sci.* **1**, 761 (1966).
10. J. H. Hildebrand and R. L. Scott, *The Solubility of Non-Electrolytes* (Dover, New York, ed. 3, 1964).
11. H. Smith, *Organic Reactions in Liquid Ammonia* (Wiley, New York, 1963).
12. I. E. Muskat, *J. Amer. Chem. Soc.* **56**, 693 (1934).
13. R. K. Robins, personal communication, July 1967.
14. A. L. Myers and J. M. Prausnitz, *Ind. Eng. Chem. Fundamentals* **4**, 209 (1965).
15. Supported by NIH grant GM 10851-11.
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Infrared Color Photography: Selective Demonstration of Bacteria

Abstract. *A microscopic procedure employs infrared color photography to enable visualization of unstained microorganisms in natural habitats. The procedure can be used for either living or dead microorganisms; it enables visualization of microbial cells embedded within component materials of the habitat.*

Visualization *in situ* of indigenous microorganisms in natural habitats, such as soil or marine muds, has proved difficult because of the inability of the microscopist to distinguish between microorganisms and colloidal humic materials and clay particles of similar size and shape; and stains often accumulate on habitat surfaces so as to preclude visualization of embedded microorganisms. These statements apply regardless of whether unstained soil is observed by phase microscopy, or stained soil is observed by light microscopy (*1*). These difficulties can now be overcome by use of Kodak Ektachrome Infrared Aero film, type 8443, to photograph the unstained organisms as wet mounts in their natural habitats.

The material to be examined is either suspended in water on a microscope slide and covered with a cover slip, or first allowed to dry on a microscope slide before resuspension and addition of the cover slip. The latter procedure can be used to decrease microbial motility. The slide is placed on the stage of a microscope having apochromatic objectives and a 12-volt conventional microscope light source; a green photographic filter is placed in the light path beneath the condenser. The condenser is lowered and the light intensity is decreased so that focusing can be accomplished on particulate matter on the microscope slide, although individual bacterial cells often cannot be seen because of the absence of a stain. An area of interest on the slide is brought into focus, the condenser is raised to a position just beneath the slide, and, in place of the green filter, either a yellow Tiffon No. 12 or a medium-red 25A Ednalite filter is placed in the light path below the condenser. The light output is then increased to 5 amp, and photographs are taken at 0.01 second, a speed fast enough for photography of moving microbial cells. The viewing magnification is 1560, while magnification at the film plane is slightly greater than 500. The film is processed with Kodak Ektachrome E-3 or E-4 chemicals without infrared inspection.

Bacteria in the resultant color transparencies appear in red "false" color, while all other inorganic and organic