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Biological Implications of Gas Chromatography

Improved instrumentation and methods increase the utility of gas chromatography to the biologist.

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In the 11 years since James and Martin (*1*) first described the analysis of fatty acids by gas chromatography, this method has earned a prominent place in the armamentarium of the analytical chemist. Many different kinds of compounds can be chromatographed; mixtures of closely related compounds can be separated rapidly, and each component of the mixture can be identified with a high degree of certainty; only exceedingly small quantities are required for a complete quantitative analysis; and the whole process can conveniently be made almost completely automatic. Because of these features, gas chromatography may be used in a number of different ways in studies of living systems. It has made many studies possible for the first time, and in other kinds of studies it completely replaced earlier methods.

Because of the great number of compounds that may be analyzed by gas chromatography, it is difficult to survey, in a limited space, all of the many applications that are potentially of interest to the biologist. In this article, therefore, I attempt, rather, to explore in some depth the capabilities of the method that make it so widely applicable, and I consider only a few applications as illustrations of these capabilities.

As in each of the other kinds of chromatography, separation of materials by gas chromatography is accomplished by subjecting the components of a mixture to repeated partition between a moving phase and a stationary phase. What distinguishes gas chromatography from the others is that the moving phase is a gas. The stationary phase may be either an adsorbing solid, as in gas-solid chromatography, or a nonvolatile liquid coated on a solid, as in gas-liquid chromatography. The partitioning process takes place within a tubular column, through which gas flows at a constant rate. The mixture to be analyzed is introduced at one end of the column. Because of the volatility of the components of the mixture, each component tends to enter the gas phase and be carried along with the carrier gas. Because of the physical and chemical attraction between the molecules and the stationary phase, the molecules also tend to be retained by the stationary phase. As a result of a balance between these effects, only a fraction of the molecules of any substance is in the gas phase at any instant.

The molecules in this fraction are carried along in the gas phase by the flowing carrier gas. In an efficient column they are carried for only a short distance before they again enter the stationary phase, and the molecules of each component can thus undergo partitioning between the two phases many times in the course of passing through the column.

In each partitioning process, the more volatile components in the mixture are partitioned in such a way that relatively larger fractions of the molecules are in the gas phase at any instant. Consequently, these components are carried through the column and out of it more rapidly than the less volatile components. If the conditions are held constant, a compound can be identified by the speed with which it passes through the column.

If a large number of partitions occur, small differences in volatility may be sufficient for separation of the compounds. The number of partitions that occur within a relatively short length of the gas-chromatography column is apparently quite large. The usual practice is to estimate an equivalent of this number by comparing the separations obtained on a column with the separations obtained in a countercurrent process. A given column is thus said to have the equivalent of a given number of theoretical plates if it separates compounds as well as a countercurrent process with that number of plates does. The length of a gas-chromatography column equivalent to a theoretical plate is usually appreciably less than 1 millimeter.

The column in which the chromatographic separations occur consists of a glass, metal, or plastic tube about 5 millimeters in diameter and from 1.2 to 3.6 meters long, or a capillary tube from 0.1 to 1.5 millimeters in diameter and up to 1½ kilometers long. When the capillary column is used, the stationary phase is most often a nonvolatile liquid coated on the column wall. When the tube of larger diameter is used it is filled with either adsorptive solid or inert solid material coated with nonvolatile liquid.

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The carrier gas is forced through the column under pressure. The carrier gas may be either an inert gas, such as helium, argon, or nitrogen, or a gas that participates to a greater extent in the partition process, such as steam, ammonia, or carbon dioxide. In either case its primary purpose is to carry the vapors of the substances being analyzed through the column.

Because of the effect of temperature on vapor pressure, the entire column is enclosed in an insulated oven, and the temperature of the oven is controlled closely. While for many analyses the column is kept at a single temperature, analysis of a group of materials that have a wide range of boiling points is often more conveniently accomplished by increasing the temperature during the course of an analysis. Automatic temperature-programming controllers are available, to help keep the rate of temperature increase reproducible.

Since the volatility of a given compound within the column is determined by the stationary phase as well as by the column temperature, the order in which components emerge from a column may be varied considerably by changing the stationary phase. Components in a mixture, therefore, can be made to emerge from the column in an order different from the strict order of their boiling points, and components with identical boiling points may often be widely separated on emerging.

Upon leaving the column, the gas is conducted to a detector, which provides an electrical signal proportional to the concentration, in the carrier gas, of the compound being analyzed. There are a number of kinds of detectors available, and each offers advantages under special circumstances.

The electrical output of the detector is usually recorded continuously, as a function of time, on a strip chart. When a detector is used that provides a signal proportional to the concentration of material in the carrier gas, the record of the analysis takes the form of a plot of concentration versus time. The emergence of a compound from the column is indicated by a gaussian-shaped peak on this record. If the carrier gas flows at a constant rate, the area under each curve represents the concentration integrated with respect to volume, or the total quantity of the component in question. Automatic integrators for determining the area under each curve are available.

From the foregoing brief description of the process, it should be apparent that the only stringent requirement a compound must fulfill in order to be analyzable by gas chromatography is that it have an appreciable vapor pressure. It must have this appreciable vapor pressure at a temperature that is lower than the temperature at which it decomposes.

Since many kinds of compounds fulfill this requirement and it is often possible to synthesize volatile derivatives of many other compounds that do not, the applicability of gas chromatography is quite wide. It is difficult to find a class of organic compounds to which gas chromatography has not been applied, and inorganic compounds of several classes have also recently been analyzed.

Analysis of Mixtures of Closely Related Compounds

The high resolution offered by gas chromatography makes the technique eminently suitable for analyzing many mixtures of closely related compounds that are very difficult to separate and analyze quantitatively by other means. The analysis of long-chain fatty acid esters is an example, which also illustrates the variety of separations that can be achieved with different stationary phases, and the sensitivity of the various detectors. Since the fatty acid composition of tissues or serum extracts is often determined under experimental conditions that are favorable for the application of gas chromatography, analysis of this family of compounds also illustrates the best features of the gas-chromatography method.

Figure 1 is a record obtained in an analysis of a mixture of fatty acid methyl esters; a capillary column was used, in which Apiezon L vacuum-stopcock grease was the stationary phase. The fully saturated fatty acid esters emerged from the column in the order of their chain lengths. Each unsaturated acid, being more polar and having a lower boiling point than the saturated acid of the same chain length, emerged ahead of the corresponding saturated acid. Methyl stearate, the fully saturated 18-carbon fatty acid ester, was retained on the column longer than mono-unsaturated methyl oleate, which was in turn retained longer than di-unsaturated methyl linoleate.

When ethylene glycol adipate polyester, a more polar material, is used

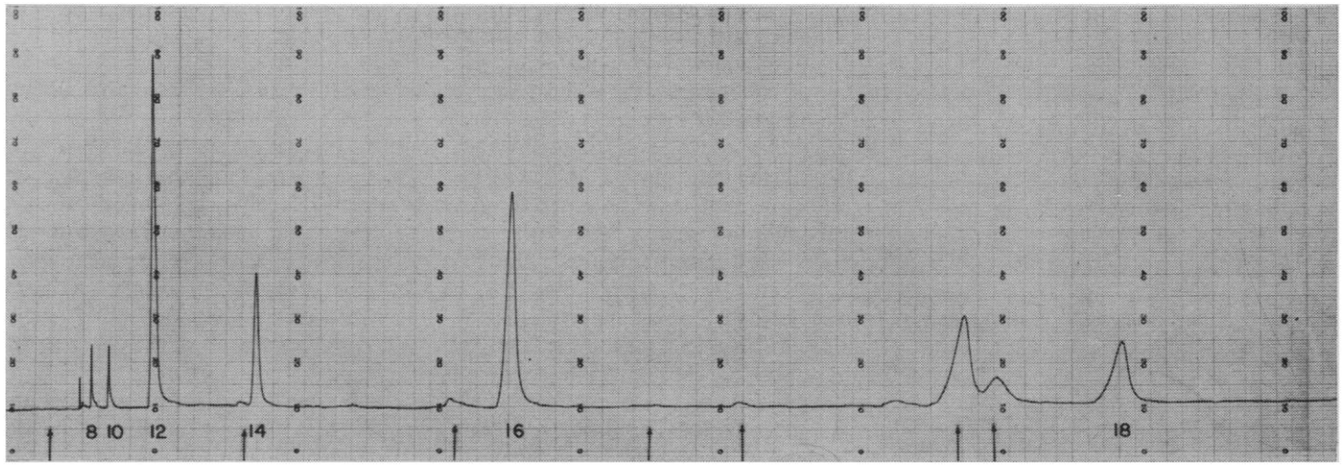

Fig. 1. Record obtained in analysis, by gas chromatography, of methyl esters of fatty acids on a capillary column (*24*). The stationary phase was Apiezon L. The detector was a radio-frequency gas discharge in helium at atmospheric pressure (*25*). The total sample was 1 microgram.

as the liquid phase, the sequence of emergence of the esters is reversed. The more polar, more unsaturated esters are retained longer than the less unsaturated esters. Figure 2 is a record obtained in an analysis of the methyl esters of the fatty acids of corn oil. The saturated, mono-unsaturated, and di-unsaturated 18-carbon esters emerged from the column in that order. The degree of separation can be varied still further by choosing a different polyester. Use of ethylene glycol succinate results in a greater separation between saturates and unsaturates than is obtained with ethylene glycol adipate polyester, for example—so much greater that the 18-carbon tri-unsaturate is retained as long as, or longer than, the 20-carbon saturated ester.

Since columns are available by means of which fatty acid esters that differ only slightly in molecular weight or chemical configuration can be separated, identification of a peak on a chromatogram is usually not difficult. Identification of individual peaks can often be confirmed by performing the analysis on more than one kind of column.

A number of highly sensitive detectors have been developed for use in gas chromatography, and many of these are applicable to the analysis of long-chain fatty acid esters. Some detectors are nonspecific in that they respond to changes in density of the gas, or to the number and size of the molecules, with little regard for the chemical nature of the substances. Among these detectors are thermal conductivity detectors, the gas density balance (*2*), detectors based on the speed of sound in the gas (*3*), and the ionization cross section detector (*4*). In general, these detectors are particularly useful in analyzing mixtures of compounds of chemically different kinds. Other detectors are highly specific for given classes of compounds. Organic acids, for example, may be detected by passing the effluent gas through a solvent and titrating the solvent continuously for acid. Similarly, halogenated compounds may be detected by subjecting the effluent to combustion, then passing it through solvent and titrating the halogen coulometrically. Still other detectors are sensitive to whole classes of compounds. The ionization detectors are examples of this kind. The hydrogen-flame ionization detector (*5*) is very sensitive to hydrocarbons and to the carbon atoms on an organic molecule that are bound to hydrogen. It is less sensitive to the carbon atoms that are bound to oxygen and is insensitive to such gases as oxygen, carbon dioxide, nitrogen, and the rare gases.

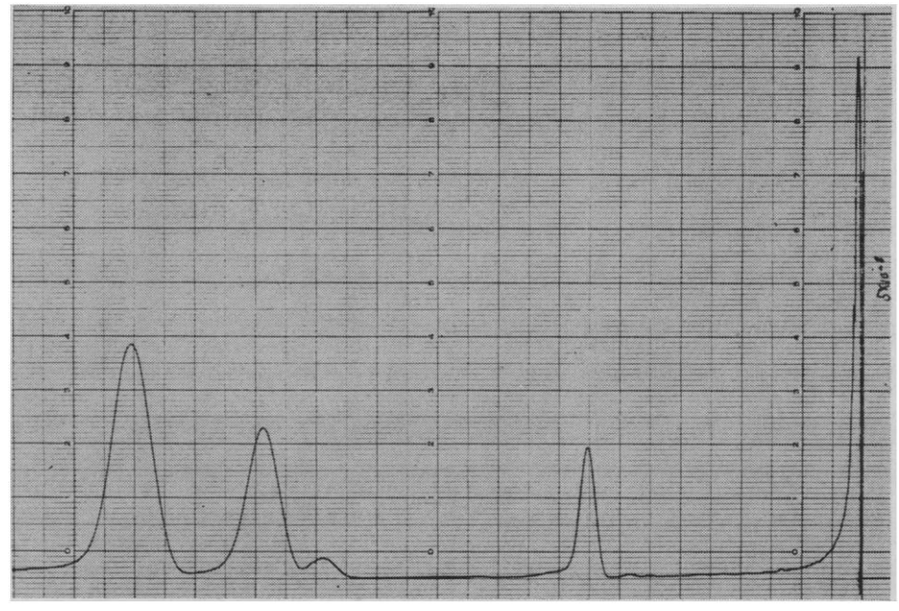

Fig. 2. Record obtained in analysis, by gas chromatography, of the methyl esters of the fatty acids of corn oil on a packed column. The stationary phase was an ethylene-glycol adipate polyester; the detector, a direct-current gas discharge in argon (*21*) at atmospheric pressure. The total sample was 3 micrograms.

The sensitivity of the specific detectors to changes in temperature is generally less than that of the nonspecific detectors, and the utilizable sensitivity is greater. Because of their specificity, however, the responses vary considerably from compound to compound, and the response of the detector to each compound in a mixture must be known if the composition is to be determined accurately.

Although the different methyl esters differ enough in chemical structure to be separable on the column, they are so similar that most detectors do not markedly discriminate among them. When any of the nonspecific detectors are used, the composition of a mixture of methyl esters can be fairly accurately determined directly from the relative areas of the peaks on the chromatogram. Because of the great difference in molecular weight between these compounds and the commonly used carrier cases, the sensitivity of the nonspecific detectors is high, and as little as 0.1 milligram of a mixture may be used for an analysis. This sensitivity, however, is very low as compared to the sensitivity of the ionization detectors, with which an accurate quantitative analysis can be obtained with from 1/100 to 1/1000 as much sample. The responses of the ionization detectors to the different esters are also grossly similar, and the areas of the peaks can often be used as approximate measures of the weights of the fatty acid esters present. The responses to the different esters are different, however, and in more accurate work the response to each fatty acid in the mixture must be determined and calibration factors must be derived.

The response of the hydrogen-flame ionization detector can be predicted approximately from the chemical formula of the ester (*6*). This detector responds to the carbon atoms in the fatty acid ester molecules that are bound to hydrogen or carbon but not to the carboxyl carbon. We can thus derive calibration factors by calculating the ratio of the formula weight of the ester to the formula weight of the ester minus the formula weight of the carboxyl. Multiplying this ratio by the area of the corresponding peak gives a numerical value approximately proportional to the weight of the ester present. The use of this calibration factor becomes increasingly significant when the sample to be analyzed contains both shorter- and longer-chain acids.

The responses of ionization detectors other than the hydrogen-flame ionization detector are less easy to predict.

For purposes of this discussion, these detectors may be considered to include all those by which materials are detected through the changes they produce in the electrical conductivity of the helium, nitrogen, or argon carrier gas, regardless of the means by which the gas is made to conduct electricity —whether by placing a source of radioactivity in the gas; by exposing the gas to ultraviolet light; or by placing it in a high-intensity electrostatic field. The response of each of these detectors results from a combination of several different effects occurring simultaneously. These effects include ionization of the material being detected by the impact of electrons; transfer of energy from excited states of the carrier gas to the materials being detected; and change in the energy and mobility of the electrons themselves. The response of each detector to any substance is a function of the current through the gas, the applied voltage, the geometry of the electrodes, and the composition of the gas. It is this last factor that makes predicting the response of any of the detectors particularly difficult. At any given voltage and current, and with any given detector geometry, the sensitivity of an argon ionization detector (*7*) to methyl esters of fatty acids is decreased by the presence of trace quantities of nitrogen, oxygen, water, and oxygenated organic materials in the argon. Diffusion of air into the detector cell may thus markedly change its sensitivity. In turn, the rate of diffusion of air into the detector varies with the rate of flow of gas through the cell and the resulting pressure of gas within it, the design of the gas outlet and insulator mounting, and the care with which the cell was constructed.

The sensitivity of the detector may also be changed by changes in column temperature if the rate of decomposition of the liquid phase changes with temperature. A polyester column, for example, may add appreciable quantities of ethylene glycol to the carrier gas as the polyester decomposes, and this may diminish the sensitivity of the detector to fatty acid methyl esters. The carrier gases used may also vary somewhat, from one batch to another, in content of water vapor and contaminants. Finally, the sensitivity of the detectors also changes with increasing concentrations of the material being detected, so that the range of concentrations within which the detector response is linearly related to concentration is limited.

The relative importance of these effects varies somewhat from detector to detector, as does the detector sensitivity, but none of the detectors is entirely immune to any of them. Because of the multiplicity of these factors, it is usually more convenient, as well as more accurate, to determine the sensitivity of a given detector empirically rather than to attempt to predict its response. Pure samples of methyl esters of fatty acids and known mixtures of different methyl esters are available for calibration.

Since the methyl esters of long-chain fatty acids can ordinarily be prepared free of water, it is not necessary to use a detector that is insensitive to water. On the other hand, the detector need not be sensitive to water. Since the ionization detectors that are less sensitive to water, atmospheric gases, and temperature are more sensitive to fatty acid ester vapor than other types of detectors, they are often preferred.

In my experience, the sensitivities obtainable with any of the ionization detectors are roughly comparable. With any of these detectors we usually use, for analysis in a packed column, samples of mixtures of methyl esters that weigh about 30 micrograms, but we could do as well with 3 micrograms and almost as well with 0.3 microgram.

There are various reasons why analysis of the fatty-acid composition of biological specimens by gas chromatography is a particularly advantageous application of the technique. These include the following.

1) The lipids, of which the fatty acids form a part, are usually easily separated by chemical extraction from the other constituents of the tissues, and quantitative conversion of the fatty acids to methyl esters is usually easily performed. The mixtures to be chromatographed are thus composed chiefly of methyl esters, mixed with only small quantities of materials that might interfere with the analysis.

2) Long-chain fatty acid esters, including those with as many as 24 carbon atoms, may be analyzed at temperatures of 200°C or lower. Materials are available for constructing detector cells that will operate at 200°C; these materials include sources of radioactivity and electrical insulators. A reasonably good selection of liquid phases that are stable at 200°C is also available, and columns may be operated continuously for many weeks. Most important of all, the long-chain methyl esters themselves are quite stable at 200°C, so that when a methyl ester is injected into a gas-chromatography column, no quantitative loss will occur before it emerges.

3) Last, and probably most significant, is the ease with which quantitative analyses of these compounds may be accurately carried out with a variety of detectors, as described earlier.

While none of these facilitating factors is essential for the analysis of a given compound by gas chromatography, the significance of each of them is best appreciated when the analysis of other classes of compounds, that do not have these properties, is compared with the analysis of fatty acid esters and it is seen that most of these other compounds are not so easily, reliably, or accurately analyzed. That so many other classes of compounds can be analyzed, even under restricting conditions, attests only to the versatility of the technique.

Analysis of fatty acids in the form of their methyl esters is an example of the use of chemical derivatives of a compound to facilitate analysis of the compound by chromatography. Free fatty acids can be analyzed as such, but because of the reactivity of the carboxyl group, care must be taken to see that the liquid phase is acid, to prevent the formation of soaps; the solid phase must be specially treated to minimize adsorption of the fatty acids; and higher temperatures must be used, because the free fatty acids are less volatile than their corresponding esters. Figure 3 is a record obtained in an analysis of free fatty acids.

Short-chain fatty acids are in some ways even more difficult to analyze. These compounds, often found in aqueous solution, may be difficult to extract and recover from such solutions without significant loss of the more volatile acids by evaporation. For the same reason, it may also be difficult to prepare less reactive derivatives of the acids. As in the case of the long-chain acids, the short-chain acids may be analyzed directly if special care is used in the preparation of the stationary phase. Since water in the sample would interfere with the detection of the acids if a nonspecific detector were used, the analyses are more easily performed with the hydrogen-flame ionization detector, which is insensi-

tive to water. The acids may then be analyzed by injecting samples of the acid in aqueous solution directly. However, this detector has a different sensitivity to each of the various short-chain fatty acids, which depends on the relative contributions of the carboxyl and the hydrogen chain of the molecule to the total weight. The response of the detector to each of the acids must be determined, and appropriate calibration factors must be used.

Other classes of compounds have so little vapor pressure, even at high temperature, that they may be chromatographed only if volatile derivatives can be prepared. The analysis of amino acids and the analysis of sugars are examples. Columns and techniques similar to those used in the analysis of long-chain fatty acid esters may be used for analysis of amino acids if the carboxyl groups of these compounds are esterified and if the amino groups are acylated or special precautions are taken in the preparation of the stationary phase (*8*). The analysis of the trimethyl silyl ether derivatives of the simple sugars on the same columns has recently been intensively investigated (*9*).

The possibility of using gas chromatography for analyzing amino acids and sugars has attracted great interest, partly because of the speed of analysis by gas chromatography but particularly because only small samples are required to obtain a complete quantitative analysis. The highly sensitive ionization detectors are therefore often preferred to any of the less sensitive nonspecific detectors. However, the amino acids as a group include compounds that vary considerably from each other in molecular weight and in oxygen and nitrogen content, and, as a result, the responses of the ionization detectors to the derivatives of one or another amino acid vary considerably. It is therefore necessary to calibrate the response of the detector for each derivative, and this requirement has markedly slowed the development of analysis of amino acids by gas chromatography. Derivatives in pure form, for purposes of calibration, usually must be synthesized in the laboratory and are not very stable. Even when pure derivatives are available for calibration it is often difficult to determine whether a given peak is small because the sensitivity of the detector to the derivative is low or whether part of the sample was decomposed or retained on the column. The response of any detector to any compound can be determined accurately by preparing C^{14}-labeled derivatives and using the radioactivity in the column effluent as a measure of the quantity of derivative reaching the detector. To my knowledge this has not been done to any great extent in connection with the analysis of amino acids by gas chromatography. One cannot say at this time that the analysis of amino acids with ionization detectors has been demonstrated to be quantitatively reliable.

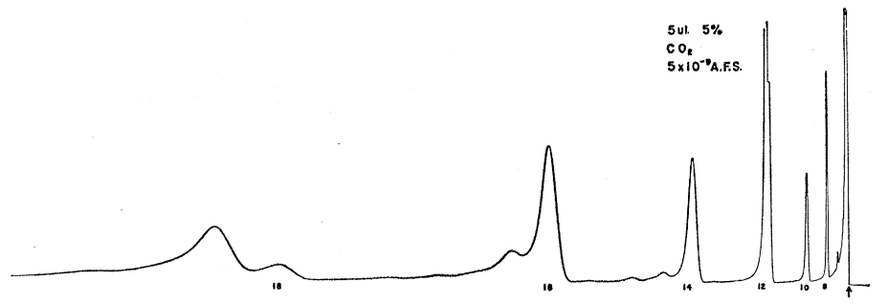

Fig. 3. Record obtained in analysis, by gas chromatography, of free fatty acids on a packed column. The stationary phase was an ethylene glycol adipate polyester. The detector was a hydrogen-flame ionization detector (*5*).

The difficulties encountered point up the continuing need for highly sensitive, nonspecific detectors to aid in making quantifying analyses of mixtures of compounds, such as the amino acids, that are essentially different chemically despite the fact that their molecules have a chemical functional group in common. An alternative solution to this problem is to use a detector that is selectively sensitive to the common functional group but is insensitive to the remainder of the molecule. The response of this detector would be proportional to the molar concentration rather than to the weight concentration of the compounds detected. Detectors based on the principle of titrating the column effluent for acid or base would fill these requirements but are not sufficiently sensitive. Unfortunately, very few selectively sensitive detectors with sensitivity comparable to that of an ionization detector are available.

The same result can be achieved if derivatives of the compounds to be detected can be synthesized by means of reagents that impart to the derivative a characteristic to which a detector is sensitive. For example, tritium- or carbon-labeled derivatives of amino acids can be prepared by acylating the amino groups with tritium- or C^{14}-labeled acetic anhydride. The radioactivity in each component of the mixture, which can be determined by a number of methods, is then proportional to the number of equivalents of amino groups present.

Still another means of accomplishing the same result is to synthesize a halogenated derivative, for analysis with a detector specifically sensitive to halogen. Many halogenated compounds, as well as several other classes of compounds, when in the vapor state, tend to form negative ions upon collision with electrons. Since negative ions are appreciably less efficient than electrons in conducting electricity in gases, very low concentrations of these compounds in a gas will markedly reduce its electrical conductivity. The electron-affinity detector (*10*) is based on this principle. Picogram quantities (1 pg = 10^{-12} g) of certain chlorinated compounds are detectable with this detector; this is about 1/1000 the minimum amount detectable with the other ionization detectors.

Trifluoroacetyl derivatives of amino acids are highly volatile and chromatograph well, but the sensitivities with which fluorine-containing compounds are detected with the electron-affinity detector is low as compared to the sensitivities with which derivatives containing other halogens are detected. Unfortunately, the amino acid derivatives that contain halogens other than fluorine are considerably less volatile than the fluorine-containing derivatives. Despite low volatility, several of the dinitrophenyl amino acid esters have been successfully chromatographed (*11*). These compounds are also markedly electronegative and may be detected with a very high degree of sensitivity by electron-affinity techniques. Landowne and Lipsky reported (*12*), however, that they found the sensitivity of the electron-affinity detector to monosubstituted and disub-

stituted dinitrophenyl amino acids to be the same—a finding which indicates that the use of this combination of derivative and detector has not yet resulted in a detector with a molar or micromicromolar response.

The analysis of steroids by gas-liquid chromatography has also been intensively studied (*13*). As a group, the steroids have high molecular weight and low vapor pressure even at high temperatures. To minimize their retention on the columns, very low concentrations of the liquid stationary phase are generally used. More than the usual amount of care in preparing the stationary phase is, therefore, frequently required to reduce the number of uncoated, active, adsorptive sites on the solid phase. Since only small quantities of materials can be analyzed with these thinly coated columns without overloading them and reducing their resolving power, highly sensitive ionization detectors are frequently required for these analyses.

The steroids differ appreciably among themselves in chemical structure and oxygen content, and the sensitivities of the ionization detectors to the various steroids differ. The factors that influence the sensitivity of the argon ionization detector to these compounds have been studied (*14*), and the conclusion has been reached that the sensitivity of the detector to a given steroid is reduced in proportion to the oxygen content of the steroid—a conclusion which, again, emphasizes the desirability of calibrating the detector for each compound.

Quantification of the results of steroid analyses made with any detector is complicated further by the reactive nature of many of the steroids. When C^{14}-labeled steroids were analyzed, it was found on several occasions that small quantities of steroid were retained on the column even though larger quantities were chromatographed as expected. This retention resulted in a disproportionately low response to small samples with each of the ionization detectors tested, and it was therefore necessary to calibrate the response of the column-and-detector combination rather than to calibrate the response of the detector alone.

The analyses of long-chain fatty acids, long-chain fatty acid esters, short-chain fatty acids, amino acids, sugars, and steroids by gas chromatography are only a few examples of the use of this method to analyze biologically significant mixtures of closely related compounds. The simplicity of the method has made many kinds of studies possible. In studies of fatty acid metabolism, for example, gas chromatography has made it possible to determine the fatty acid composition of serum lipids in each of the various classes, and thus to study the factors that influence the composition of these lipids. Similarly, gas chromatography has made it possible to study the fatty acid composition of various tissues and the relationship between this fatty acid composition and that of the various serum lipids. A great variety of studies of the factors that influence the fatty acid composition of tissues are being carried out throughout the world by means of this technique.

In other biochemical and physiological studies, information about the rates of synthesis and the pathways of the metabolism of compounds is desired. The dynamic aspects of the metabolism of many compounds can be studied by using C^{14}- and tritium-labeled compounds in conjunction with methods for measuring carbon-14 and tritium in the effluent of a gas-chromatography column. We can thus analyze a mixture of components, determining which components are radioactive and the specific radioactivity of each. Any of several methods of measuring the radioactivity can be used, depending on the quantity of radioactivity in the sample. Figure 4 is a record obtained in an analysis of fatty acid esters derived from a lipid fraction from the lymphatic duct of a rat that had been fed C^{14}-labeled palmitic, stearic, oleic, and linoleic acids in the course of a study of fatty acid absorption. The upper record was obtained in a quantitative analysis of the fatty acid esters as detected by an argon ionization detector. The lower record shows the carbon-14 in these acids as detected by scintillation counting (*15*). Analyses such as these enabled us to determine the specificity for fatty acid of the mechanism for synthesizing cholesterol ester and phospholipid in the course of intestinal absorption of fatty acid (*16, 17*).

This analysis was performed by passing the effluent of the column through a combustion tube in which the fatty acid esters were burned to carbon dioxide and water, then through a water trap, and finally through finely divided anthracene crystals for carbon-14 assay by scintillation counting. The assay for radioactivity was thus made essentially as the gas left the column. When the radioassay is made in this way, the time in which the radioactivity can be measured is limited, since, if too much time is taken, the resolution of the analysis is reduced. The time taken for the radioassay is set by the volume of the anthracene detector cell and the rate of flow of gas through it. Since a delay of up to 15 seconds can usually be tolerated in an analysis of methyl esters without reducing the resolution, the rate of flow and the volume of the detector are chosen accordingly. The sample should contain enough radioactive material for the radioactivity to be measurable in this short period. When it contains less than this amount, the effluent of the column can be fractionated, the fatty acid esters can be condensed out of the gas stream, and each fraction can be assayed for radioactivity for a much longer period. Automatic fraction collectors have been developed to aid in performing radioassay by this second method (*18*).

The ability to measure the radioactivity in the effluent of a gas chromatography column should also prove useful as an ancillary specific method for detecting compounds leaving the column, as described earlier in the discussion of analysis of amino acids by gas chromatography. This kind of specific detector has already proved useful in another application of gas chromatography—measurement of the quantities of specific compounds present in very small amounts in complex biologic extracts.

Gas Chromatography as a Method of Microassay

The high sensitivity of the gas chromatography detectors suggests the use of gas chromatography for measuring the levels of trace components in biologic extracts. Measurement of pesticide residues in foodstuffs is one application of gas chromatography that is particularly promising; measurement of steroid hormones in serum and urine is another. The difficulties encountered in analysis of the amino acids because of the differences in chemical structure in that family of compounds are accentuated in the measurement of pesticides or steroids, since the extracts almost always contain mixtures of many different kinds of compounds that tend to interfere with the analyses. Thus, although a highly sensitive detector is

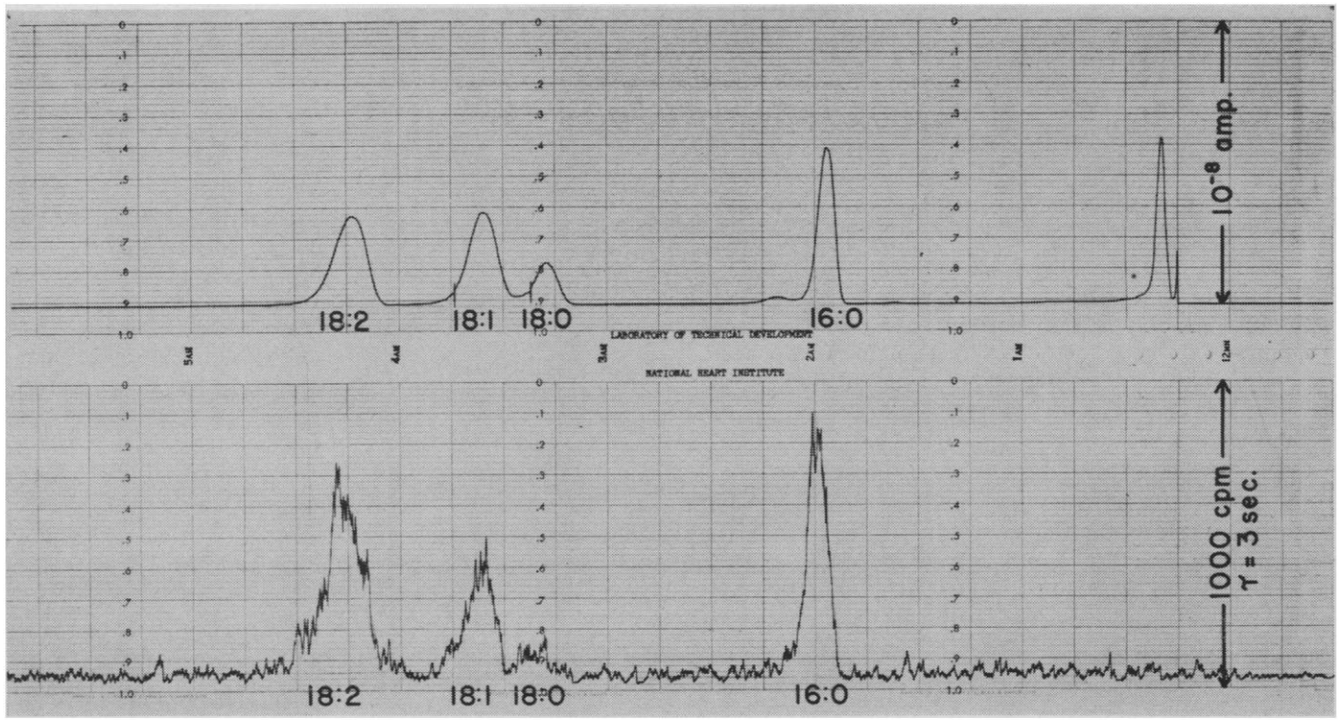

Fig. 4. Record showing the radioactivity in the effluent of a gas-chromatography column. The upper record gives a quantitative analysis of all the fatty acids present. The lower record shows the carbon-14 radioactivity in these acids, as detected by scintillation counting.

necessary to detect the trace quantities of pesticide or steroid present, a detector that offers a degree of specificity of response as well as high sensitivity is often even more useful.

Because of the high sensitivity of the electron-affinity detector to halogenated compounds, it is hoped that this detector will be particularly useful in analyses of chlorine-containing pesticides. Since plant extracts contain a large variety of compounds that behave much as the insecticides do in the course of gas chromatography, the extract ordinarily must be purified considerably by chemical and other chromatographic methods before it can be analyzed by this means. The electron-affinity detector is relatively insensitive to many classes of compounds, so the amount of purification that is necessary is appreciably reduced.

The problems encountered in developing methods for assay of steroid hormones and their metabolites in body fluids are similar in that these compounds are usually found in low concentration in the extracts, mixed with many other kinds of compounds in high concentration. We are less fortunate in this instance in that no detector specifically sensitive to steroids is available.

Although many methods have been developed for separating a great number of steroids in synthetic mixtures, we cannot yet say that these methods have proved applicable to routine assay of steroids in biologic extracts in many instances. All too often the analyses of extracts show the presence of many unidentified compounds as well as small peaks tentatively identified as the steroids being sought. In such analytical records it is often difficult to determine what part of a given peak is attributable to an interfering substance and what part to the compound of interest. This difficulty is clearly attributable to the lack of a selectively sensitive detector.

The techniques described earlier in connection with the analysis of amino acids (techniques such as the synthesis of derivatives of the compounds for which selectively sensitive detectors are available) may be applicable here. To test the feasibility of assaying testosterone in this way, C^{14}-labeled testosterone acetate was prepared by causing testosterone to react with C^{14}-labeled acetic anhydride. By measuring the radioactivity in the testosterone acetate peak, nanogram quantities (1 mg = 10^{-9} g) of testosterone could be detected (*19*). This detection method is sensitive only to those compounds in an extract that have chemical groups that react with acetic anhydride and would be completely insensitive to those that do not. A similar approach in which chloroacetyl derivatives and the electron-affinity detector are used has been described (*20*).

Gas Chromatography for Monitoring Composition of a Changing Mixture

Analyses by gas chromatography can be performed very rapidly, especially if very high resolution is not required, and this made the technique useful for monitoring the composition of a large mass of material as an aid in controlling a continuous chemical process. Automatic equipment for securing and injecting samples into the column repetitively has been developed. Modifications of such equipment should be useful for monitoring levels of anesthetic gases in gas mixtures. Along these lines, many analytical separations of potential interest to the physiologist have been described, as well as many separations that are of interest to workers in other fields.

Gas chromatography has been used in monitoring the composition of respiratory gases, flue gases, automobile exhausts, and atmospheric pollutants; in analyzing alcoholic beverages, per-

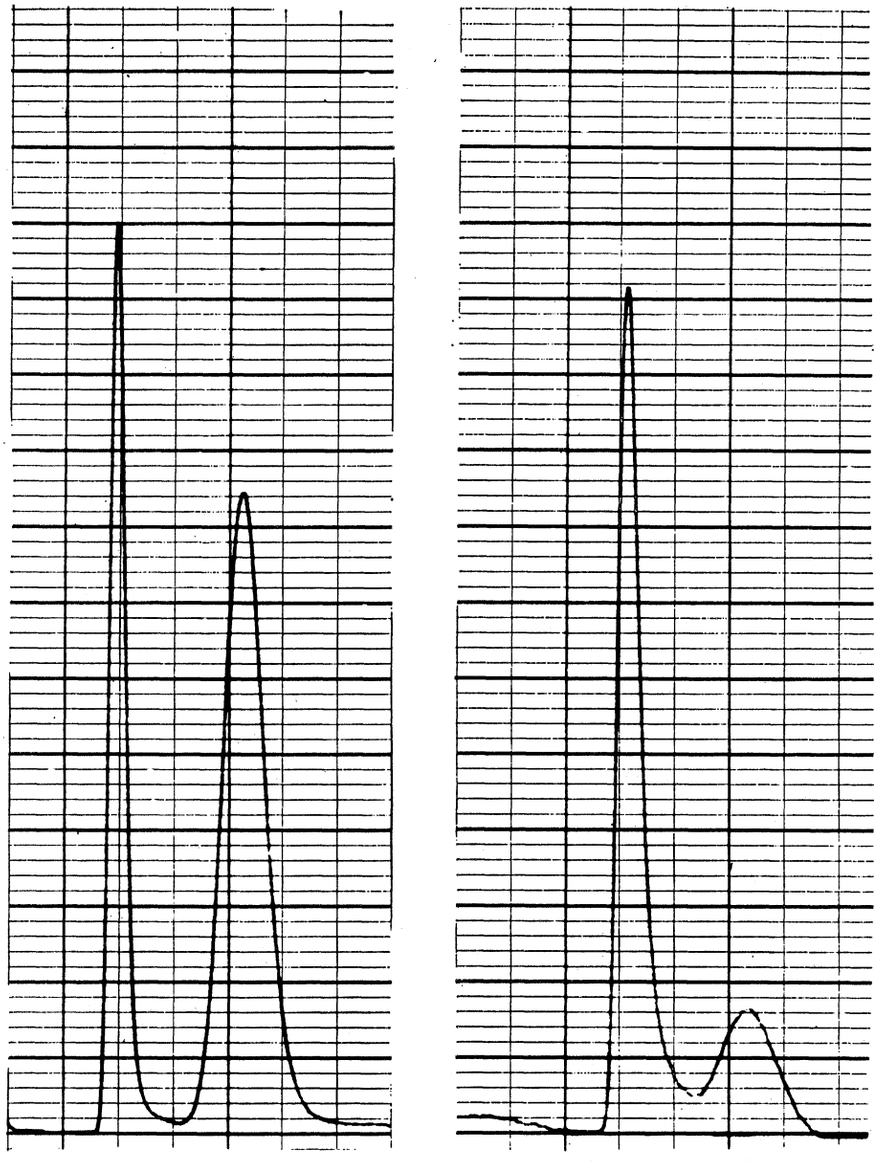

Fig. 5. Separation of 1-microliter samples of air into oxygen and nitrogen by a molecular sieve column, as detected by the effects of these gases on (left) a direct-current discharge in helium, and (right) a direct-current discharge in argon.

fumes, and the aromas and tastes of foods; and in studying the senses of smell and taste.

Just as we could choose among several detectors in the analysis of materials with high boiling points, we have a choice in monitoring different kinds of mixtures. Figure 5 is a record obtained in the separation of oxygen and nitrogen in samples of air by a molecular-sieve column. Two different ionization detectors were used, one with a direct-current discharge in helium and one with a direct-current discharge in argon (*21*). Both of these detectors are more sensitive to oxygen than to nitrogen. This is reflected in the relatively large size of the oxygen peak on both the records of Fig. 5. Although the areas under the curves cannot be used directly for quantifying the analysis unless the detector response is calibrated for each gas, the areas under each peak can readily be used to follow changes in concentration and in relative concentrations when repeated analyses of mixtures of these two gases are made. The ionization detectors are useful here particularly because their high sensitivity makes it possible to perform the analyses more rapidly than they could be performed with less sensitive detectors.

It is interesting to contemplate, as an alternative and somewhat better method of monitoring the composition of simple mixtures, the possibility of using two-dimensional gas chromatography in the same way that two-dimensional continuous electrophoresis is used for analyzing mixtures. The material to be monitored would be delivered to the column continuously rather than repetitively, and each compound would emerge from the column at a specific location and be detected by a detector mounted at that location. However, a convenient method for causing the materials on the gas-chromatography column to move in the second dimension is needed to make this approach feasible.

Blood Gases

The rapidity with which gases such as oxygen, nitrogen, and carbon dioxide can be separated from each other encouraged many workers to apply gas chromatography for the measurement of these gases in blood. The problems encountered have centered around the method for extracting the gases from the blood and effecting the transfer of known quantities of the extracted gases into the gas-chromatography column. A vacuum extraction apparatus, similar to that used in more conventional measurements of gases in blood, has been used (*22*). A number of simpler devices have also been tried. The difficulties encountered by most workers suggest that, in general, it is easier to use gas chromatography to determine the relative composition of a mixture than it is to use it to determine the quantity of a single compound in a solution, such as the concentration of oxygen in blood. Methods for injecting materials into the gas-chromatography column in known quantity have not been developed to the same degree of precision as some of the other techniques used in connection with gas chromatography.

Other Applications of Gas-Chromatography Detectors

Since many of the advantages offered by gas chromatography are the direct result of the highly sensitive detection methods, the possibility of adapting gas-chromatography detectors for use in other forms of analysis seemed worthy of investigation.

Many compounds not sufficiently volatile for analysis by gas chromatog-

raphy can readily be separated by thin-layer chromatography or by liquid-liquid chromatography. We tested the feasibility of using a gas-chromatography detector for quantitative analysis of such compounds. Solutions of nonvolatile lipids in volatile organic solvents, such as might be found in the effluent of a liquid-liquid-chromatography column, were injected into a very short section of tubing filled with diatomaceous earth, in the way that mixtures are introduced into a gas chromatography column. Nitrogen was pumped through this tubing and then led to a hydrogen-flame ionization detector. After the solvent had evaporated, the temperature of the column was increased rapidly; this caused the nonvolatile lipids to be pyrolyzed. The volatile products of pyrolysis were delivered to the hydrogen-flame ionization detector. Microgram quantities of lipids with high boiling points, in volatile solvents, were measurable in this way (*23*).

The high sensitivity of the gas-chromatography detectors also prompted us to explore other uses for them, apart from chromatography. The hydrogen-flame ionization detector has been used without a gas column for monitoring the total hydrocarbon content of air samples. It is eminently suitable for this because of its insensitivity to oxygen, nitrogen, water vapor, carbon dioxide, and rare gases. Still another possible application is the use of a gas-chromatography detector, without a column, to monitor the concentration of volatile organic materials in aqueous streams. For this application, a method of separating or stripping the organic material from the bulk of the water is needed. A sampling device that was found to accomplish this very well was constructed by mounting a thin silicone rubber membrane over the end of a tube made of polytetrafluoroethylene. Through a tube of smaller diameter in the bore of the first tube, gas was delivered to the membrane, blown over its surface, and then led to a hydrogen-flame ionization detector through the unfilled bore of the outer tube. Any volatile substance that passed through the membrane was thus delivered to the detector. When the membrane was immersed in solutions of ether in water, the detector response was proportional to the concentration of ether in the water.

This application of a gas-chromatography detector is feasible because the silicone rubber membrane is selectively permeable to organic materials and the hydrogen-flame ionization detector is insensitive to water vapor. Several different kinds of membrane can be used, as well as several different kinds of detector, to achieve different relative sensitivities for specific applications. We also used the electron-affinity detector in conjunction with a small silicone rubber membrane to detect chloroform and halothane in water, and we achieved an appreciable increase in sensitivity. The concentration of water vapor that can be tolerated by this detector is limited, however.

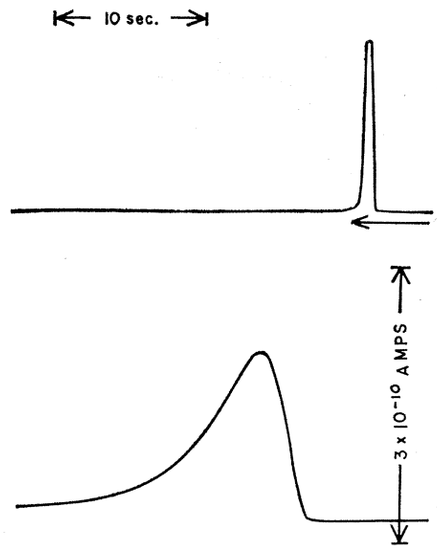

Fig. 6. The concentration, as a function of time, of diethyl ether in pulmonary arterial blood of a dog after the injection into a femoral vein of 2 milliliters of normal saline saturated with ether. The concentration was sensed by the passage of ether through a sampling device consisting of a silicone rubber membrane inserted within the vein. The pulse on the upper graph was recorded at the time of injection. The response of the hydrogen-flame ionization detector is recorded in the lower graph.

This method of detecting volatile organic compounds in aqueous solution has many possible applications. It was of particular interest to us because of the possibility of using the membrane as an intravascular detector for materials dissolved in blood. The ability to monitor the concentration of injected foreign substances at various points in the circulatory system would be very useful in studies of cardiovascular dynamics and in the detection of abnormal circulatory pathways. An experiment was performed in which a silicone rubber membrane, 2 millimeters in diameter, mounted over the end of a long tube, was placed in the pulmonary artery of a dog. Figure 6 shows the response of the hydrogen-flame ionization detector when a 2-milliliter sample of saline saturated with diethyl ether was injected into the femoral vein.

Summary

In summary, the potential significance of gas chromatography to the biologist is great. It makes possible the quantitative analysis of certain classes of volatile compounds, such as the long-chain fatty acids, that differ only slightly from each other in chemical and physical structure, and thus makes possible many metabolic studies not otherwise possible. The resolving power of the columns is so high and the convenience with which the quantitative analysis may be obtained is so great as to encourage continuation of the search for methods of making derivatives of essentially nonvolatile compounds, such as amino acids and sugars, that will make it possible to analyze these compounds, too, by gas chromatography. By labeling a substance to be studied with radioactive isotopes, one can also use gas chromatography to study the dynamics of the metabolism of many compounds. The high sensitivity of the detectors makes gas chromatography useful in assaying for specific compounds present in very small concentrations in plant extracts and body fluids. At the same time, the difficulties encountered in developing these assay methods emphasize the continuing need for more specific, highly sensitive methods of detection. Finally, consideration of the methods used in gas chromatography suggests ways by which other analytical techniques might be improved, including the possibility that the detectors developed for gas chromatography might be used more extensively for other purposes.

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Electric Propulsion

For space exploration we need rockets that are better, not necessarily bigger, than those being developed.

W. E. Moeckel

Propulsion is a means of producing motion, or, more accurately, changes in momentum. Propulsion is also required to maintain momentum in the presence of resistive or retarding forces. These functions of a propulsion system require the expenditure of energy. If this propulsive energy must be carried by the vehicle, as is the case with most systems, then the amount of change in momentum, or the length of the period during which a given momentum can be maintained, is determined by the form and amount of the energy carried along. Ideally, this energy should be in a form which yields the maximum useful propulsive energy per unit of weight.

Other factors, however, such as development and operating problems and costs, or the adequacy of existing systems, may argue against use of systems having the highest possible energy per unit weight. In the case of aircraft, for example, the use of nuclear power would have greatly increased the energy per unit weight, but it involved many difficult and costly problems of development and operation. Its advantages, in terms of much greater range and duration of flight, were overbalanced by these disadvantages, and by the fact that kerosene-burning aircraft were already available with range, speed, and flight durations which were adequate for most applications of interest.

For space missions, if man would be satisfied with exploring the moon, or even with settling a colony on it, there would be little incentive to develop propulsion systems with much greater energy per unit weight than the chemical rockets now in use or under development. Some economies might eventually be realized, it is true, if nuclear rockets or electric rockets were to be developed, but on the other hand, there are many improvements that might be made in chemical rockets, such as using the air through which the vehicle passes to enhance the useful energy, recovering and re-using the launching vehicle, or developing chemicals of higher energy. The net gain in using nonchemical energy sources for spacecraft on lunar missions would therefore be marginal, as it is for nuclear-propelled aircraft on terrestrial missions.

However, if man wants to travel beyond the moon, the need for propulsion systems with more energy per unit weight than chemical rockets becomes painfully obvious. To launch the Mercury spacecraft into orbit required a launching vehicle with about 163,000 kilograms (~ 360,000 lb) of thrust, standing about as high as a seven-story building. To launch the Apollo spacecraft to the moon will require a launching vehicle (a Saturn 5) with about 3.4 million kilograms of thrust, standing about as high as a 30-story building. To send an expedition of seven men to explore Mars and return would require a launching vehicle with thrust of about 68 million kilograms, standing higher than a 70-story building.

Now, no one is seriously proposing to launch an expedition to Mars with a single monstrous launching vehicle. Instead, parts of the interplanetary vehicle could be launched into a low orbit around the earth, and the pieces could be assembled in orbit. Even this, however, becomes a project of tremendous magnitude, even if high-energy reactants such as hydrogen and oxygen are used. It would involve 20 to 40 launchings with the huge Apollo booster (Saturn-5), and the total weight of the orbiting vehicles would be between 2 and 4 million kilograms. The propellant would account for more than 90 percent of the initial weight at launch and also for more than 90 percent of the weight of the orbiting vehicle. To reduce this weight significantly, the useful propulsive energy of the propellant must be substantially increased.

Useful Propulsive Energy

To get the most useful propulsive work out of each bit of propellant, the propellant must be ejected rearward at the highest possible speed, because high rearward momentum of the propellant implies, according to Newton's law, a high forward thrust on the vehicle. Useful propulsive energy is, therefore, the kinetic energy of the ejected propellant, and this energy depends only on the exit velocity.

As we have seen, the chemical energy of combustion is not enough to produce, with a reasonable weight of propellant, the ejection speeds (more commonly called jet velocities) needed for manned interplanetary missions. There are two alternative energy sources: nuclear energy and solar energy.

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