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The Carbon-Fluorine Bond in Compounds of Biological Interest

Studies with fluorinated molecules can be helpful in understanding biological phenomena.

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Recent advances in organic fluorine chemistry have been responsible for the development of a large number of new compounds of importance in biology and medicine. Perhaps this new technology has been of greatest concern to the toxicologist who has been compelled to evaluate the biological effects of the large number of new fluoro compounds used as insecticides, refrigerants, and aerosol propellants (*1*). However, the knowledge gained in the synthesis of organic fluorine compounds has also provided the pharmacologist with selective inhibitors of biological processes and has given the medicinal chemist the opportunity to design more active therapeutic agents. With increased interest in fluoro compounds, traditional views on the extraordinary stability of the carbon-fluorine bond have been modified as a result of the finding that this bond is cleaved in a number of different enzymatic reactions. The use of fluoro compounds in studies of enzyme and pharmacological mechanisms has advantages not found with many other analogs since insight into the biochemical phenomenon can often be gained from an understanding of the altered chemical properties conferred on the compound by the fluorine substituent.

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Relevant Properties of Fluorine

Fluorine is in group VII of the periodic table, but this fact should be considered in light of Pauling's reference to it as a "superhalogen" (*2*). This description can be appreciated from the data of Table 1 which show that the properties of the other halogens place them in a group distinct from fluorine. Fluorine is considerably more electronegative than the other halogens, and for this reason it is the only halogen which is extremely unlikely to form the positive ion (F^+) (*3*). The bond energy of the carbon-fluorine bond is among the highest found in natural products or known to be broken enzymatically.

The van der Waals' radius of the fluorine atom (1.35 angstroms) is closest in size to that of hydrogen (1.1 angstroms). Most of the other substituent groups often used to replace hydrogen in the creation of analogs are much larger (see Table 1 for sizes of the other halogens; methyl, trifluoromethyl, and phenyl groups are also large). Thus fluorine is of unique value in the design of analogs which can very closely approach the natural biochemical intermediate. Good analogs of this kind can be useful therapeutically but they can also be extremely valuable in defining critical sizes that contribute to structural considerations in biochemically important molecules.

These properties of fluorine are of most interest to the biologist since a knowledge of the size and electronegativity of the atom can be used to make defined alterations in biologically important molecules. Studies based on these properties of fluorine are governed often by the characteristics of the carbon-fluorine bond which determine the reactivity of such molecules in biological systems.

Metabolic Effects of Fluoro Analogs

Discussion of the role of organic fluorine compounds in biology should begin with the fascinating story of fluoroacetate (*4*). Although the toxic properties of this compound had been known earlier, it was not until 25 years ago that fluoroacetate was found as a natural product in *Dichapetalum cymosum*. This discovery by Marais (*5*) not only explained the toxicity of these plants to grazing cattle in South Africa but initiated a period of research on the actions of fluoroacetate which has had many ramifications in the development of biochemical pharmacology. Studies in the laboratories of Peters and Martius made it clear that fluoroacetate toxicity in mammalian tissues was related to an inhibition of citrate metabolism, the inhibition being caused by a factor, distinct from fluoroacetate, which could be isolated after tissues had been incubated with fluoroacetate. This inhibitory factor proved to be fluorocitrate (*6*) which acts as an inhibitor of the Krebs cycle. Fluorocitrate is synthesized from fluoroacetate by a pathway analogous to that of the synthesis of citrate from acetate, as shown in Fig. 1. In view of the similarity in size between the fluorine and hydrogen atoms, it is not surprising that some enzymes can metabolize fluoroacetate instead of acetate.

The actions of fluorocitrate as an inhibitor of the Krebs cycle are complex as indicated by Fanshier *et al.* (*7*). These workers have extended the evidence that only one of the four iso-

mers of fluorocitrate made by organic synthesis is inhibitory; as expected, this corresponds to the isomer made enzymatically by the pathway of Fig. 1. Furthermore, they have shown that succinic dehydrogenase as well as aconitase is inhibited by fluorocitrate. Aconitase seems to undergo competitive inhibition only if initial rates are examined, since there is also a progressive time-dependent inactivation of the enzyme by fluorocitrate (*7*).

The complex interaction of fluorocitrate with aconitase may indicate an additional value of fluoro analogs, namely, in the elucidation of the detailed relation between proteins and small molecules. Such a possibility is suggested by the finding that only one of the four isomers of fluorocitrate seems to react with the enzyme; data on the properties of these isomers might provide exact knowledge of the features of citrate which are required for the interaction and, by inference, some notion of the configuration of the protein at the reactive site. In contrast to the studies with aconitase are studies on the allosteric activation of acetyl coenzyme A (acetyl-CoA) carboxylase by citrate. In this system fluorocitrate (containing all four isomers) is as effective as citrate (*8*); this indicates that different structural aspects of citrate are responsible for its interaction with this protein.

This digression onto the possibilities for further research with fluorocitrate should not overshadow the impact that this compound has already had on our thinking in pharmacology. Peters has coined the term "lethal synthesis" (*4*) to emphasize that the toxic compound fluorocitrate appears only as a result of the metabolic transformation of the relatively nontoxic fluoroacetate. Such a mode of action has been found in a number of pharmacologically active agents and is of fundamental importance in the design of new therapeutic agents.

The finding of ω-fluoropalmitate and ω-fluorooleate in the seeds of *Dichapetalum toxicarium* (*9*) suggests that fluoroacetate can substitute for acetate, at least partially, in the biosynthesis of long-chain fatty acids. The fact that fluorine occupies the ω-position of the fatty acid is in accord with the scheme for fatty acid biosynthesis which has now been demonstrated in animals, plants, and microorganisms (*10*). In all of these systems acetyl-CoA is the 2-carbon unit responsible for elongation of the fatty molecule. Yet in the completed straight-chain fatty acid molecule, only the ω-carbon and the adjacent carbon atom are derived directly from acetyl-CoA; the other acetyl-CoA molecules are carboxylated to form malonyl-CoA and are then decarboxylated in the condensation reaction. Figure 2, which portrays the initial cycle of condensation and reduction in the 2-carbon elongation of a fatty acid molecule, illustrates the dual role of acetyl-CoA in this process. Acetyl-CoA, which proceeds through malonyl-CoA (shown with the hypothetical X substituent), undergoes reactions not required of acetyl-CoA entering the methyl terminal position (shown as fluoroacetyl-CoA). Failure of fluoroacetyl-CoA to proceed through "fluoromalonyl-CoA" would account for the lack of incorporation of fluorine except at the ω-position of the fatty acid product. The finding of fluorine exclusively at the ω-position may also occur because a substituent at the 2-position in the condensed product (Fig. 2) interferes with the reductive steps required after the condensation reaction. Furthermore, since the synthesis of long-chain fatty acids is a repetitive process, partial inhibition at any step would be expected to have a large effect on the overall synthesis.

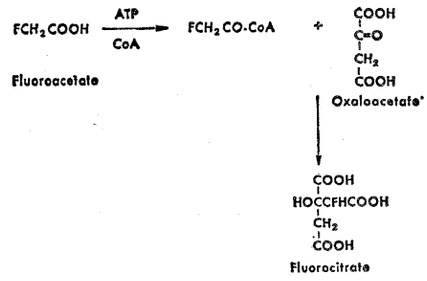

Fig. 1. Pathway for the synthesis of fluorocitrate from fluoroacetate. ATP is adenosine-5′-triphosphate.

The acceptability of fluoroacetyl-CoA in the initial condensation reaction is in accord with results of studies on fatty acid synthesis in an enzyme system obtained from adipose tissue. In this enzyme system a number of acyl-CoA compounds can substitute for acetyl-CoA in the initiation of fatty acid synthesis (*11*). Such a substitution for acetyl-CoA yields a product which differs from the natural one simply by a change in the acyl group substituted for acetate at the ω-position. In other words, the subsequent reactions with malonyl-CoA seem not to be greatly affected by the acyl group initiating the condensation. Considerations of this kind might explain the finding of ω-fluoro fatty acids in the seeds of *Dichapetalum toxicarium* and the apparent small incorporation of fluoroacetate into the lipids of rats fed sublethal doses of this compound (*12*).

Saunders, Pattison, and their coworkers have shown that ω-fluoro fatty acids containing an even number of carbon atoms have a toxicity to rats comparable to that of fluoroacetate, whereas ω-fluoro acids with an odd number of carbon atoms are strikingly less toxic (*13*). Such findings are in accord with β-oxidation of fatty acids in which the successive loss of 2-carbon acetyl-CoA units would yield either the toxic fluoroacetyl-CoA, if there are initially an even number of carbon atoms, or the relatively nontoxic 3-fluoropropionate, if there are initially an odd number of carbon atoms in the ω-fluoro fatty acid. The toxicity of fluoro compounds can indicate whether the biological degradation produces fluoroacetate, and this often can provide a clue to metabolism of compounds in which the fluorine serves as a marker. For example, in a series of ω-fluoronitriles, $F(CH_2)_nCN$, those with an odd number of carbon atoms are toxic, whereas those with an even number are nontoxic (*14*); these observations suggest a carbon-cyanide cleavage rather than a hydrolysis or reduction of the nitrile in the metabolism of these compounds. Examples of what might be termed lethal catabolism can also occur in microorganisms. A pseudomonad, which can utilize nicotinate for growth, cannot grow on 5-fluoronicotinate, presumably because of the accumulation of fluoroacetate and fluorocitrate (*15*). Studies on the degradation of 2-fluoronitrobenzoic acid by *Nocardia erythropolis* (*16*) have made the identification of these toxic products more definitive.

The principles learned in the study of fluoroacetate have been extended to nucleic acid metabolism by Heidelberger and his co-workers who realized that fluorinated pyrimidines (synthesized chemically) might provide a means of interfering with the cellular synthesis of nucleic acids (*17*). This approach to blocking nucleic acid synthesis as a method of cancer chemotherapy seemed particularly attractive since uracil utilization is greater in tumors than in normal tissues. Of the compounds developed in this approach the most useful have been 5-fluorouracil and 5-fluoro-2-deoxyuridine, both of which depend for their effectiveness on

metabolic transformation within the cell to an active form, namely, 5-fluorodeoxyuridine monophosphate. In this form, the fluorinated pyrimidine becomes an inhibitor of thymidylate synthetase (*18*), an enzyme responsible for the methylation of deoxyuridine monophosphate to yield thymidine monophosphate, which is required as a precursor in the synthesis of DNA. This example of lethal synthesis is analogous to that of fluoroacetate, since 5-fluorouracil is converted to its active form by a normal metabolic pathway, in this case that responsible for the conversion of uracil to deoxyuridine monophosphate. In the form of 5-fluorodeoxyuridine monophosphate, the fluorine of the fluorouracil becomes responsible for blocking a step in the overall pathway leading to the synthesis of DNA. Tumors resistant to 5-fluorouracil can have a low level of an enzyme on the pathway from uracil to deoxyuridine monophosphate or a resistant thymidylate synthetase and are thus protected from the formation or action of the active chemotherapeutic agent (*19*). Fortunately for the usefulness of these compounds, the metabolic degradation of 5-fluorouracil in vivo yields the relatively nontoxic α-fluoro-β-alanine (*17*) rather than a fluorine compound with other toxic properties.

The unique value of fluorine in the design of analogs is illustrated by the fact that 2-fluoroadenosine is strikingly more toxic for cultured cells than the other 2-halogenated adenosines. The basis for this difference apparently lies, at least in part, in the specificity of adenosine kinase, which phosphorylates 2-fluoroadenosine more readily than adenosine but is unreactive with the other 2-halogenated adenosines (*20*).

Another aspect of the toxicity of these substituted adenosines might be their ability to conform to the geometric restrictions inherent in the structure of polynucleotide helices. It is likely that 2-fluoroadenosine would conform to these restrictions in contrast to homologous compounds with bulkier substituents, such as bromine or iodine, which would not meet these criteria. Studies based on these structural considerations have been made in a series of 8-substituted purines. Acs (*21*) has shown that a number of 8-substituted purines (including 8-bromoadenosine), which are not toxic for cultured fibroblasts, are also not substrates for the adenosine kinase. On the other hand, the toxic analog 8-aminoadenosine is phosphorylated but is not incorporated

Table 1. Some properties of the halogens (*2*).

Halogen	van der Waals' radius (Å)	Electronegativity	Bond energy (kcal/mole)	
			To carbon	To hydrogen
F	1.35	4.0	107	147.5
Cl	1.80	3.0	66.5	102.7
Br	1.95	2.8	54.0	87.3
I	2.15	2.4	45.5	71.4

(less than 1 percent) into RNA or DNA by polymerases in vitro. On the basis of model building studies, it is uncertain whether the moderately small amino substituent could conform to the restrictions at the 8-position of the purine imposed by the DNA structure. Kapuler and Reich (*21*) have shown that 8-bromo and 8-keto guanosine triphosphate (GTP) are not incorporated into polymers by replicating enzymes. They note, however, that the smaller fluorine atom at the 8-position of the purine might satisfy the conformational requirements necessary for incorporation. The fluorinated compound would therefore have considerable value in defining the restrictions imposed by the helical structures and in establishing criteria for toxicity. If the 8-fluoro purines were incorporated by polymerases, the fluorine substituent could resemble the hydrogen atom sufficiently to permit the substitution to occur without toxicity.

Questions of this kind serve to emphasize the particular value of fluorinated analogs. Although these compounds may be more of a challenge to the synthetic chemist, they provide considerable insight into the biological system. It is also likely that subtle differences in biochemical intermediates that are introduced by fluorine in this way might be exploited chemotherapeutically.

The interest in fluoro analogs as selective metabolic inhibitors has inspired the development of other fluoro analogs. In Bergmann's laboratory, a systematic effort has been made to synthesize intermediates of the Krebs cycle with a fluorine substituent (*22*). Through the efforts of Bergmann and others, fluoro analogs have been developed for various glycolytic intermediates, sugars, amino acids, and cholesterol precursors (*4*). In general, the biological effects of many of these compounds have not been particularly noteworthy. However, a number of interesting specific enzyme inhibitors have been characterized, particularly by Kun and his collaborators (*7, 23*).

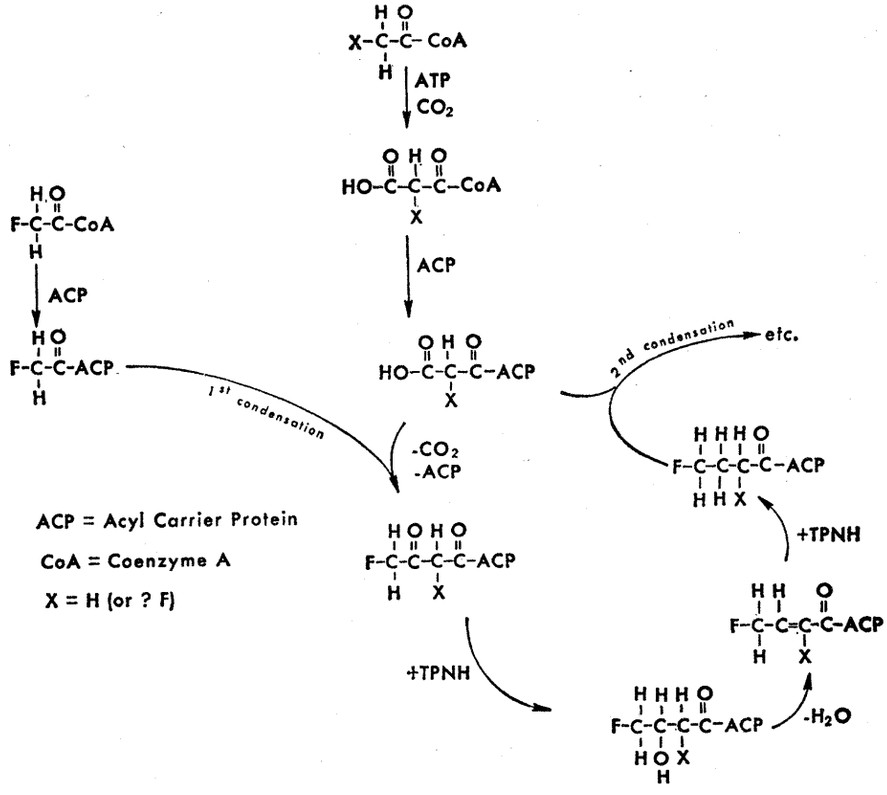

Fig. 2. Cycle in long-chain fatty acid biosynthesis showing a possible role for fluoroacetate.

Alterations in Macromolecules by Fluoro Analogs

In addition to their action as inhibitors of specific enzymes, fluoro analogs can disrupt cellular function by being incorporated into the macromolecules of the cell. Among the fluorinated amino acids incorporated into proteins are 5′,5′,5′-trifluoroleucine, 2-fluorophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, 3-fluorotyrosine, and 4-fluoroproline (*24–26*). In dissecting the role of macromolecules in cellular function, it has been useful to attribute changes in cell activity as the result of analog administration to alterations in the particular macromolecule known to be affected by the analog. For example, the limitation of viral infection in tissue culture cells when 4-fluorophenylalanine is present suggests that new proteins which are nonfunctional because of the analog must be synthesized during a particular phase of virus replication (*27*). This approach is supported by studies which indicate that an enzyme such as β-galactosidase, in which normal aromatic amino acids are replaced by a fluoro analog, has altered physical properties (*26*). The physiological effect of altered proteins after analog incorporation is suggested by experiments on mutants of *Escherichia coli* with temperature-sensitive repressors of either alkaline phosphatase or capsular polysaccharide synthesis. Such mutants make more of the particular product (are derepressed) at a lower temperature when 4-fluorophenylalanine is added to the medium. The explanation of this phenomenon seems to be that the temperature-sensitive repressor protein suffers a further critical loss in its function when it contains the analog amino acid (*28*).

In addition to inhibiting DNA synthesis, fluorouracil can be incorporated into the RNA of mammalian cells, bacteria, viruses, and phage (*29*). Both the messenger RNA (*30*) and transfer RNA (*31*) of *Escherichia coli* seem to incorporate the analog. The consequences of this can be lower infectivity of a virus, altered protein synthesis as in the case of the bacterial enzymes (*32*), or change in the phenotype of a bacteriophage (*33*). It has seemed logical to ascribe this type of alteration to translational errors arising as the result of fluorouracil pairing with guanine instead of adenine. However, the transcription errors that would be predicted when 5-fluorouridine triphosphate is substituted for uridine triphosphate have not been demonstrated experimentally (*34*). It has been demonstrated that transfer RNA undergoes changes in its physical properties as the result of the incorporation of fluorouracil and that this altered transfer RNA does not function normally in an amino acid–incorporating system under the direction of the RNA of bacteriophage R17 (*31*). These results suggest that altered transfer RNA may contribute to the altered cell function arising from the incorporation of fluorouracil into RNA. Changes in physical properties of RNA have also been found when fluorouracil is incorporated biosynthetically into the RNA bacteriophage MS2. The RNA particles containing fluorouracil are not infectious although they can serve as an active template for the synthesis of phage-specific protein in cell extracts (*34a*).

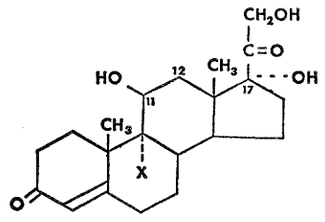

Fig. 3. Hydrocortisone with X indicating the substituent at the 9α-position.

Another aspect of the relation of fluoro analogs to macromolecules comes from studies of Rennert which indicate that 5′,5′,5′-trifluoroleucine can be incorporated into the protein of mouse embryos but not into that of the mature organism. A difference in the amino acid–activating enzymes and in the transfer RNA seems to be responsible for this difference (*35*). The altered response to analogs of the immature tissue may be an important clue to a biochemical basis of differentiation. Such a study also illustrates the value of analogs in the elucidation of biochemical differences between tissues. These differences are of the same type as those sought by the chemotherapist.

Table 2. Relation of adrenal cortical activity to the electronegativity of the substituent at the 9α-position of hydrocortisone (*36*).

Substituent (X)	Glycogenic activity of 9α-X-hydrocortisone	K_a of XCH_2COOH $\times 10^5$
Fluorine	10.7	217
Chlorine	4.7	155
Bromine	0.3	138
Iodine	0.1	75
Hydroxyl	0.2	16
Methoxyl	<0.2	33
Hydrogen	1.0	1.82

Structure-Function Relations

Interest in fluorination of the steroid molecule has increased our knowledge of the chemistry of fluorination reactions and has given additional insight into features of the steroid molecule that are responsible for its biological activity. With these advances has come the development of steroids which are more active therapeutic agents. It is not possible in a short review to detail all of the structure-function relations in the steroid molecule, but it is hoped that one example from the studies of Fried (*36*) will illustrate the usefulness of fluorine in this field of medicinal chemistry. Hydrocortisone (Fig. 3) requires an 11β-hydroxyl group for its biological activity. This activity, which can be measured as an effect on increasing liver glycogen in the rat, can be correlated with the nature of various substituents at the 9α-position as shown in Table 2.

If the activity of the substituent were related to its size, one would expect the fluorine substituent to have about the same effect as the hydroxyl or hydrogen group, and similarly chlorine to have the same effect as the methoxyl substituent. Clearly this is not the case, and for this reason the table also includes a measure of the electronegativity of the substituent. This is demonstrated by the effect of the substituent in increasing the acidity of substituted acetic acids.

In this series, the greater the constant K_a, the more readily the substituent withdraws electrons and the more easily a proton is released from the neighboring carboxylic group. The data indicate that biological activity of the steroid molecule correlates quite well with electronegativity as measured in this manner; this correlation suggests that biological activity may be related to the acidity of the 11β-hydroxyl group caused by the substituent. The prediction of increased biological activity due to increased acidity of the 11β-hydroxyl group is further supported by studies of a family of compounds in which biological activity and the acidity of the hydroxyl group are altered by substitutions at the 12-position of the molecule (*36*).

Because of its electronegativity, a fluorine substituent can stabilize more labile adjacent carbon-halogen bonds in a molecule. The use of CF_3I containing I^{131} as a radioactive inert gas for the measurement of local cerebral blood flow (*37*) illustrates this, since the parent compound CH_3I is quite unstable in biological systems. A field in which systematic substitution of a fluorine atom in various compounds has been helpful in elucidating relations between structure and activity is the pharmacology of general anesthetic agents (*38*).

Enzymology of the Carbon-Fluorine Bond

In general, the usefulness of fluorine in the development of interesting pharmacological agents has been related either to its size, which is comparable to that of hydrogen, or to its electronegativity, which affects the acidity or stability of neighboring groups. Implicit in the importance of fluorine for these purposes, as illustrated by the studies mentioned above, is the extraordinary stability of the carbon-fluorine bond in biological systems. Indeed, because of the difficulty encountered by the analytical chemist in cleaving this bond, it had been questioned whether the bond could be broken enzymatically. Fluoroacetate illustrates this stability since fluoride is released only slowly from this compound when it is refluxed in 20 percent sodium hydroxide or heated at 100°C in concentrated sulfuric acid. Complete release of fluoride occurs only in sodium fusion at 400°C or after refluxing in 30 percent sodium hydroxide (*39*).

Subsequently, two examples of the enzymatic cleavage of the carbon-fluorine bond were found. The first is the action of horseradish peroxidase on 4-fluoroaniline (*40*). Cleavage of the carbon-fluorine bond in this case is not unique since chloro, iodo, or methoxy substituents at the 4-position of an aniline compound are displaced in analogous reactions by the enzyme. The removal of fluoride from another aromatic carbon compound occurs by the action of phenylalanine hydroxylase on 4-fluorophenylalanine. Several possible schemes have been advanced by Kaufman (*41*) to explain this reaction which yields stoichiometric amounts of fluoride and tyrosine from 4-fluorophenylalanine at the expense of excessive triphosphopyridine nucleotide (reduced form) (TPNH) and presumably molecular oxygen. Aside from the abnormal stoichiometry of the reaction with 4-fluorophenylalanine, there is nothing to suggest that this reaction differs from the normal reaction of the enzyme with phenylalanine. It is not clear how any known mechanism can explain the cleavage of the carbon-fluorine bond by these two enzymes.

Since studies of peroxidase and phenylalanine hydroxylase did not particularly clarify any unique aspects that might exist in the enzymology of carbon-fluorine cleavage, it seemed that additional insight into this problem might be gained from studies of other enzymes capable of catalyzing this type of reaction. Since fluoroacetate was a natural product (*42*), it seemed likely that, in spite of its great stability, there might be enzymes capable of degrading it. Such an enzyme could possibly be found if fluoroacetate were used as a carbon source in an enrichment culture.

In enrichment (or elective) culture, a microorganism which is capable of utilizing a particular compound as a sole source of carbon or nitrogen, or both, is selected from soil. When isolated and grown in pure culture, such an organism can provide a convenient system for studying the metabolism of the compound. Many metabolic pathways, enzyme mechanisms, and cofactors that were later shown to have a general importance in biochemistry were first noted in microorganisms isolated by this method.

An enrichment culture containing fluoroacetate as a sole source of carbon was successful in yielding a pseudomonad which contained an enzyme capable of breaking the carbon-fluorine bond in this compound (*43*). The ease of isolating such an organism is perhaps emphasized by the nearly simultaneous report of similar organisms obtained by similar techniques by other investigators in Japan and the British Isles (*44*). The organism studied in our laboratory was only one of eight isolates obtained at that time. Studies with an enzyme partially purified from this organism (*43*) indicated that fluoride was released from fluoroacetate according to the following equation

$$FCH_2COO^- + H_2O \rightarrow HOCH_2COO^- + HF$$

Studies of the specificity of this enzyme indicated that fluoride is not released from a variety of other compounds containing the carbon-fluorine bond. Among the nonreactive compounds were 2-fluoropropionate and 3-fluoropropionate. Thus the enzyme is not a general one for the cleavage of carbon-fluorine bonds. Rather, tests on other substrates suggest that the enzyme is specific for the dehalogenation of monohalogenated acetic acids, for example, fluoroacetate, chloroacetate, and iodoacetate (bromoacetate was not tested). On the basis of this specificity, the enzyme was named haloacetate halidohydrolase. As shown in Table 3, cleavage of the carbon-fluorine bond is faster than that of the other carbon-halogen bonds. In addition, the Michaelis constant for chloroacetate ($2 \times 10^{-2}M$) is tenfold higher than that for fluoroacetate.

A consideration of the other substrates which react with the fluoroacetate-splitting enzyme casts a new light on the enzymatic cleavage of the carbon-fluorine bond. In spite of its status as a "superhalogen," fluoride is liberated by a mechanism which does not appear to differ from that by which the other halides are released from the analogous compounds. This similar release of halides in spite of the higher bond energy of the carbon-fluorine bond may be related to the hydrogen-halogen bond which can be considered to form during the reaction. A summary of these bond energies (Table 1) indicates that, in general, the energy of the hydrogen-halogen bond correlates with that of the carbon-halogen bond. This

Table 3. Relative reaction rates of halidohydrolases with haloacetate halidohydrolase (*H*), halidohydrolase I, and halidohydrolase II. Rate with monochloroacetate set at 100 for each enzyme. All substrates tested at $10^{-2}M$ as their sodium salts.

Substrate	Relative rate of the halidohydrolase			Nonhalide product
	H	I	II	
FCH_2COO^-	700	0	0	$HOCH_2COO^-$
$ClCH_2COO^-$	100	100	100	$HOCH_2COO^-$
L-$CH_3ClCHCOO^-$		50	120	D-$CH_3CHOHCOO^-$
ICH_2COO^-	.5	20	80	$HOCH_2COO^-$
Cl_2CHCOO^-		10	200	$OCHCOO^-$
L-$CH_3CH_2ClCHCOO^-$		10	80	D-$CH_3CH_2CHOHCOO^-$
$CH_3CCl_2COO^-$		0	0	

relation suggests that the energy lost in the cleavage of the particular carbon-halogen bond may be gained during the reaction by the formation of the corresponding hydrogen-halogen bond. The net energy change during the reaction with the carbon-fluorine bond would then be about the same or even slightly more favorable than that for the other carbon-halogen bonds.

A mechanism which invokes protonation of the halide leaving group, as suggested by the above discussion, would explain why fluorine is a better leaving group than iodine in this reaction. However, the difference in reactivity of the halogens might also be attributed to a poorer fit of the enzyme with the bulkier substituents. This latter explanation finds support in the fact that 2-fluoropropionate, which differs from fluoroacetate in the introduction of a bulky methyl substituent, is not dehalogenated.

The removal of all halides from the 2-position of a substituted acetic acid by an apparently similar mechanism suggested that additional insight into this mechanism might be gained from an investigation of other enzymes of this type. Accordingly, dichloroacetate (a component of the antibiotic chloramphenicol produced by *Streptomyces venezuelae*) was used to select another organism which might yield a similar but different enzyme. The pseudomonad isolated on this carbon source proved to be more valuable than anticipated, since the organism had the ability to produce two additional halidohydrolases, one induced during growth on dichloroacetate (halidohydrolase II), and the other during growth on chloroacetate (halidohydrolase I) (*45*). The two halidohydrolases have different physical properties and are separable by column chromatography. As indicated in Table 3, both enyzmes attack the same substrates but at different rates. The range of substrates attacked overlaps with that of haloacetate halidohydrolase, the enzymes differing in this regard only in their ability to attack fluoroacetate and certain 2-substituted halogenated acids. These three enzymes also have in common a *p*H optimum of about 9.2 (*43*, *45*).

The reactions catalyzed by the two additional halidohydrolases can be summarized by the equation

$$\text{L-RCHXCOO}^- + \text{H}_2\text{O} \rightarrow \text{D-RCHOHCOO}^- + \text{HX}$$

where X = Cl or I and R = H, CH_3, or CH_3CH_2.

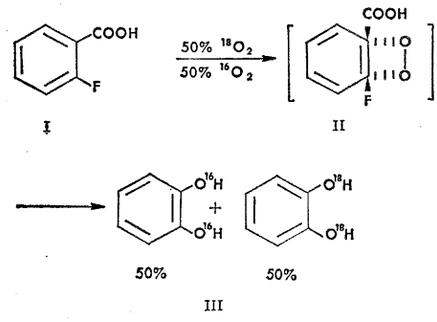

Fig. 4. The microbial metabolism of 2-fluorobenzoate (I) to 3-fluorocatechol (III) in an atmosphere which is 50 percent O^{16}-labeled O_2 and 50 percent O^{18}-labeled O_2. The proposed cyclic peroxide intermediate (II) is in accord with the oxygen labeling found in 3-fluorocatechol.

In accord with this equation, the halide in each of the substrates of Table 3 is displaced by a hydroxyl group. The apparent exception to this rule is dichloroacetate from which the product is glyoxylate. Actually, this product is also in accord with the general equation, since the expected product, namely, $HOClCHCOO^-$, is unstable and would spontaneously eliminate HCl to yield glyoxylate. As in the reactions of haloacetate halidohydrolase, these halidohydrolases displace the halide with a hydroxyl group containing oxygen derived from water (*45*, *46*). This is an important distinction between the cleavage of the carbon-halogen bond by the halidohydrolases and that in 4-fluorophenylalanine by phenylalanine hydroxylases. Although formally in each reaction the halide is displaced by a hydroxyl group, in the hydroxylase-catalyzed reaction the oxygen is derived from molecular oxygen rather than from water (*47*).

Similarities among the three halidohydrolases indicate that these enzymes can quite properly be considered to belong to the same class. Halidohydrolase I and II, however, by virtue of being reactive with optically active substrates, provide some additional insight into mechanisms involved in this type of reaction. The fact that a Walden inversion occurs during the hydroxyl displacement of the halide indicates that the enzyme catalyzes a bimolecular nucleophilic substitution reaction. This is the mechanism involved in the nonenzymatic hydroxyl displacement of a halide which occurs when these compounds are treated with sodium hydroxide (*48*).

The increased use of fluorinated compounds in enzymology has revealed other examples of the enzymatic hydrolysis of the carbon-fluorine bond. Barnett *et al.* tested α-D-glucosyl fluoride as a reagent which might form a covalent bond to the postulated carrier for sugar transport in intestinal cells. They found, however, that α-D-glucosyl fluoride was hydrolyzed enzymatically to glucose and fluoride, apparently by the enzyme α-D-glucosidase (*49*). A similar chance discovery of a defluorinating reaction was made by Henkart *et al.* during an attempt to modify carbonic anhydrase by the Sanger reagent, 1-fluoro-2,4-dinitrobenzene. They found that the enzyme catalyzes the hydrolysis of this compound, presumably with the formation of 2,4-dinitrophenol and fluoride (*50*). Sanger's reagent is quite specific as an alkylating agent for certain groups in proteins. The use of this compound probably suggested that α-D-glucosyl fluoride could be an alkylating agent for a protein expected to have a high affinity for glucose.

The use of fluorinated compounds as alkylating agents suggested that an alkylated enzyme might be an intermediate in the reaction catalyzed by haloacetate halidohydrolase (*43*). In this proposal, the reaction would proceed by the two half reactions

$$XCH_2COO^- + ESH \rightarrow ESCH_2COO^- + HX$$

and

$$ESCH_2COO^- + H_2O \rightarrow ESH + HOCH_2COO^-$$

where X = F, Cl, or I and E represents the enzyme with its sulfhydryl group designated by SH. This mechanism seemed particularly attractive since there was evidence for a sulfhydryl group on the enzyme and iodoacetate, a well-known alkylating agent, was a substrate rather than an inhibitor of the enzyme. The first half reaction is analogous to nonenzymatic reactions of sulfhydryl compounds with chloroacetate in which the halide is eliminated and a thioether is formed.

In an optically active substrate, however, an alkylated enzyme would require one inversion of configuration during the first half reaction and a second inversion in the second half reaction. In this double inversion the product would be expected to retain the configuration of the substrate. Such a mechanism would, therefore, be unacceptable as an explanation of the halidohydrolase reactions in which an inversion of configuration occurs.

Fluoride is released from 2-fluorobenzoic acid in a reaction involving

molecular oxygen that has been found in a pseudomonad isolated from an enrichment culture on 2-fluorobenzoic acid (*51*). The enzyme for this reaction has not been characterized but in whole cells the catechol derived from 2-fluorobenzoic acid has been isolated. Furthermore, when the incubation of the cells is carried out in an atmosphere containing O_2 labeled with both oxygen-16 and oxygen-18, it can be demonstrated that both oxygen atoms in the catechol are derived from a single molecule of oxygen (*52*). Although other explanations are possible, this finding suggests that the reaction proceeds through a cyclic peroxide (Fig. 4) such as has been postulated for the analogous oxidation of anthranilic acid to catechol (*53*). The microsomal hydroxylating system from rabbit liver which catalyzes the conversion of 4-fluoroaniline to 4-hydroxyaniline is another example of carbon-fluorine bond cleavage which seems to require molecular oxygen (*54*).

Except for the reaction catalyzed by peroxidase, all reactions of carbon-fluorine bond cleavage involve the replacement of the fluorine group by a hydroxyl group. These reactions differ, however, depending on whether the oxygen in the hydroxyl group is derived from water or from molecular oxygen. Oxygen has been shown to come from water in the reaction catalyzed by haloacetate halidohydrolase and oxygen almost certainly comes from water in the reactions catalyzed by α-glycosidase and carbonic anhydrase where water is the usual reactant with the enzyme. On the other hand, oxygen comes from molecular oxygen in the reaction by which catechol arises from 2-fluorobenzoate and almost certainly in the reaction of phenylalanine hydroxylase on 4-fluorophenylalanine and in the reaction with 4-fluoroaniline catalyzed by the microsomal enzyme. Presumably the defluorination of 4-fluoroproline which occurs in a collagen-synthesizing system (*25*) also requires molecular oxygen.

Although studies with these enzymes do not permit many unifying observations about the nature of carbon-fluorine bond cleavage, several aspects of the relation of fluorine to the other halogens seem worth mentioning. The specificity of the enzyme haloacetate halidohydrolase indicates that the carbon-fluorine bond can be cleaved by mechanisms which are applicable to the fission of comparable bonds involving the other halogens. On the other hand, in aromatic compounds undergoing reactions with hydroxylases, the fate of fluorine can be different from that of the other halogens. In the conversion of 4-fluorophenylalanine to tyrosine by phenylalanine hydroxylase, fluoride is released whereas in the reaction of the same enzyme with 4-chlorophenylalanine, the major product is 3-chlorotyrosine (*55*). The difference in the fate of the two halogens does not necessarily imply any fundamental difference in the enzyme mechanism whereby the two substrates are hydroxylated. Fluorine and chlorine substituents do not necessarily behave differently during hydroxylation, since in the microsomal system 4-hydroxyaniline is the product of the reaction with both 4-fluoroaniline and 4-chloroaniline (*54*).

Although considerable insight has been gained into enzymatic mechanisms of carbon-fluorine bond cleavage, the biochemistry of carbon-fluorine bond formation remains to be elucidated. By developing an in vitro system for fluoroacetate formation in homogenates of *Acacia georginae*, Peters and his collaborators have begun the attack on this challenging problem (*56*).

Summary

The principles learned from studies of fluoroacetate and the application of these principles in the use of fluorouracil tend to emphasize the value of organic fluorine compounds in the design of selective inhibitors of enzymes. This important application may obscure other uses that organic fluorine compounds can have in various areas of biological science. The alterations in the properties of a molecule that can often be predicted from a fluorine substituent are valuable in the development of correlations between structure and function, particularly when fluorine is compared systematically to other substituents on the basis of size or electronegativity. Recent studies which show the changes in physical and chemical properties of RNA and proteins after the incorporation of fluoro analogs indicate that structure-function correlations of this kind may be extended to macromolecules. A particularly challenging area with potentially wide practical application in therapeutics is the use of fluorinated analogs as probes to elucidate subtle differences in the biochemistry of different tissues. It is hoped that the recent finding of carbon-fluorine bond cleavage by enzymes will give more insight into the biochemical properties of this bond and also contribute to an understanding of the mechanism of certain kinds of enzyme catalysis. It seems clear that the availability of judiciously designed fluoro analogs will be of considerable value in the development of these approaches.

References and Notes

1. J. W. Clayton, Jr., in *Handbook of Experimental Pharmacology*, O. Eichler, A. Farah, H. Herken, A. D. Welch, Eds. (Springer-Verlag, New York, 1966), vol. 20, part 1, p. 459.
2. L. Pauling, *The Nature of the Chemical Bond* (Cornell Univ. Press, Ithaca, N.Y., ed. 3, 1960), p. 82.
3. A. G. Sharpe, *Quart. Rev.* **11**, 49 (1957).
4. R. A. Peters, *Advan. Enzymol.* **18**, 113 (1957); F. L. M. Pattison and R. A. Peters, in *Handbook of Experimental Pharmacology*, O. Eichler, A. Farah, H. Herken, A. D. Welch, Eds. (Springer-Verlag, New York, 1966), vol. 20, part 1, p. 387.
5. J. S. C. Marais, *Onderstepoort J. Vet. Sci. Anim. Ind.* **20**, 67 (1944).
6. C. Liebécq and R. A. Peters, *Biochim. Biophys. Acta* **3**, 215 (1949); C. Martius, *Justus Liebigs Ann. Chem.* **561**, 227 (1949); R. A. Peters, R. W. Wakelin, P. Buffa, L. C. Thomas, *Proc. Roy. Soc. London Ser. B Biol. Sci.* **140**, 497 (1953).
7. D. W. Fanshier, L. K. Gottwald, E. Kun, *J. Biol. Chem.* **239**, 425 (1964).
8. P. R. Vagelos, A. W. Alberts, D. B. Martin, *ibid.* **238**, 533 (1963).
9. R. A. Peters, R. J. Hall, P. F. V. Ward, N. Sheppard, *Biochem. J.* **77**, 17 (1960); P. F. V. Ward, R. J. Hall, R. A. Peters, *Nature* **201**, 611 (1964).
10. F. Lynen, *Fed. Proc.* **20**, 941 (1961); S. Wakil, *Annu. Rev. Biochem.* **31**, 369 (1962); P. R. Vagelos, *ibid.* **33**, 139 (1964).
11. M. G. Horning, D. B. Martin, A. Karmen, P. R. Vagelos, *J. Biol. Chem.* **236**, 669 (1961).
12. E. M. Gal, P. A. Drewes, N. F. Taylor, *Arch. Biochem. Biophys.* **93**, 1 (1961).
13. F. J. Buckle, F. L. M. Pattison, B. C. Saunders, *J. Chem. Soc. London* **1949**, 1471 (1949); F. L. M. Pattison and B. C. Saunders, *ibid.*, p. 2745; F. L. M. Pattison, *Toxic Aliphatic Fluorine Compounds* (Elsevier, Amsterdam, 1959).
14. F. L. M. Pattison, W. J. Cott, W. C. Howell, R. W. White, *J. Amer. Chem. Soc.* **78**, 3484 (1956).
15. E. J. Behrman and R. Y. Stanier, *J. Biol. Chem.* **228**, 947 (1957).
16. E. K. Tranter and R. B. Cain, *Biochem. J.* **103**, 22P (1967).
17. C. Heidelberger, in *Progress in Nucleic Acid Research and Molecular Biology*, J. N. Davidson and W. E. Cohn, Eds. (Academic Press, New York, 1965), vol. 4, p. 2.
18. S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, J. Lichtenstein, *Proc. Nat. Acad. Sci. U.S.* **44**, 1004 (1958); K-U. Hartmann and C. Heidelberger, *J. Biol. Chem.* **236**, 3006 (1961); P. R. Reyes and C. Heidelberger, *Biochim. Biophys. Acta* **103**, 177 (1965).
19. C. Heidelberger, G. Kaldor, K. L. Mukherjee, P. B. Danneberg, *Cancer Res.* **20**, 903 (1960); P. Reichard, O. Skold, G. Klein, L. Revesz, P-H. Magnusson, *ibid.* **22**, 235 (1962).
20. L. L. Bennett, Jr., M. H. Vail, S. Chumley, J. A. Montgomery, *Biochem. Pharmacol.* **15**, 1719 (1966); H. P. Schnebli, D. L. Hill, L. L. Bennett, Jr., *J. Biol. Chem.* **242**, 1997 (1967).
21. G. Acs, personal communication; A. M. Kapuler and E. Reich, personal communication.
22. E. D. Bergmann, *Bull. Res. Counc. Israel* **10A**, 1 (1961).
23. E. Kun, J. E. Ayling, B. G. Baltimore, *J. Biol. Chem.* **239**, 2896 (1964); J. E. Ayling and E. Kun, *Mol. Pharmacol.* **1**, 255 (1965); R. J. Dummel, E. Kun, S. K. Roy, *Biochemistry* **4**, 2547 (1965).
24. O. H. Halvorson and S. Spiegelman, *J. Bacteriol.* **64**, 207 (1952); R. Munier and G. N.

Cohen, *Biochim. Biophys. Acta* **31**, 378 (1959); M. H. Richmond, *J. Mol. Biol.* **6**, 284 (1963); O. M. Rennert and H. S. Anker, *Biochemistry* **2**, 471 (1962).
25. A. A. Gottlieb, Y. Fujita, S. Udenfried, B. Witkop, *Biochemistry* **4**, 2507 (1965).
26. R. L. Munier and G. Sarrazin, *Compt. Rend. Ser. C* **259**, 937 (1964); *ibid.* **262**, 1029 (1966).
27. E. Wecker and E. Schonne, *Proc. Nat. Acad. Sci. U.S.* **47**, 278 (1961); L. Leventow, M. M. Thorén, J. E. Darnell, Jr., L. L. Hooper, *Virology* **16**, 220 (1962).
28. S. Kang and A. Markovitz, *J. Bacteriol.* **93**, 584 (1967); *ibid.* **94**, 87 (1967).
29. N. K. Chaudhuri, B. J. Montag, C. Heidelberger, *Cancer Res.* **18**, 318 (1958); J. Horowitz and E. Chargaff, *Nature* **184**, 1213 (1959); M. P. Gordon and M. Staehelin, *Biochim. Biophys. Acta* **36**, 351 (1959); Y. Shimura and D. Nathans, *Biochem. Biophys. Res. Commun.* **16**, 116 (1964).
30. F. Gros, W. Gilbert, H. H. Hiatt, G. Attardi, P. F. Spahr, J. D. Watson, *Cold Spring Harbor Symp. Quant. Biol.* **26**, 111 (1961).
31. R. J. Lowrie and P. L. Berquist, *Biochemistry* **7**, 1761 (1968).
32. Catabolite repression rather than fluorouracil incorporation into messenger RNA seems to be the explanation for inhibition of β-galactosidase synthesis; J. Horowitz and V. Kohlmeier, *Biochem. Biophys. Acta* **142**, 208 (1967).
33. S. P. Champe and S. Benzer, *Proc. Nat. Acad. Sci. U.S.* **48**, 532 (1962).
34. H. Bujard and C. Heidelberger, *Biochemistry* **5**, 3339 (1966).
34a. Y. Shimura, R. E. Moses, D. Nathans, *J. Mol. Biol.* **28**, 95 (1967); Y. Shimura, H. Kaizer, D. Nathans, *ibid.* **38**, 453 (1968).
35. O. M. Rennert, personal communication.
36. J. Fried, *Cancer* **10**, 752 (1957). I thank J. Fried for permission to reproduce his data in Table 2.
37. L. Sokoloff, in *New Research Techniques of Neuroanatomy*, W. Windle, Ed. (Thomas, Springfield, Ill., 1957), p. 54.
38. J. C. Krantz, Jr., and F. G. Rudo, in *Handbook of Experimental Pharmacology*, O. Eichler, A. Farah, H. Herken, A. D. Welch, Eds. (Springer-Verlag, New York, 1966), vol. 20, part 1, p. 501.
39. B. C. Saunders and G. J. Stacey, *J. Chem. Soc. London* **1948**, 1773 (1948); P. Mirosevic-Sorgo and B. C. Saunders, *Tetrahedron* **5**, 38 (1959).
40. G. M. K. Hughes and B. C. Saunders, *J. Chem. Soc. London* **1954**, 4630 (1954); B. C. Saunders, A. G. Holmes-Siedle, B. P. Stark, *Peroxidase* (Butterworths, London, 1964).
41. S. Kaufman, *Biochim. Biophys. Acta* **51**, 619 (1961); S. Kaufman, in *Oxygenases*, O. Hayaishi, Ed. (Academic Press, New York, 1962), p. 129.
42. In addition to the species of *Dichapetalum* mentioned, three other species of plants have been found to contain fluoroacetate; L. R. Murray, J. D. McConnell, J. H. Whittem, *Australian J. Sci.* **24**, 41 (1961); P. B. Oelrichs and T. McEwan, *Nature* **190**, 808 (1961); M. M. Oliveira, *Experientia* **19**, 586 (1963); T. McEwan, *Nature* **201**, 827 (1964).
43. P. Goldman, *J. Biol. Chem.* **240**, 3434 (1965).
44. N. Horiuchi, *Nippon Nogei Kagaku Kaishi* **35**, 870 (1961); K. Tonomura, F. Futai, O. Tanabe, T. Yamaoka, *Agr. Biol. Chem. Tokyo* **29**, 124 (1965); M. Kelly, *Nature* **208**, 809 (1965).
45. P. Goldman, G. W. A. Milne, D. B. Keister, *J. Biol. Chem.* **243**, 428 (1968).
46. P. Goldman and G. W. A. Milne, *ibid.* **241**, 5557 (1966).
47. S. Kaufman, W. F. Bridgers, F. Eisenberg, S. Friedman, *Biochem. Biophys. Res. Commun.* **9**, 497 (1962).
48. W. A. Cowdrey, E. D. Hughes, C. K. Ingold, *J. Chem. Soc. London* **1937**, 1208 (1937).
49. J. E. G. Barnett, W. T. S. Jarvis, N. K. A. Munday, *Biochem. J.* **103**, 699 (1967).
50. P. Henkart, G. Guidotti, J. T. Edsall, *J. Biol. Chem.* **243**, 2447 (1968).
51. P. Goldman, G. W. A. Milne, M. T. Pignataro, *Arch. Biochem. Biophys.* **118**, 178 (1967).
52. G. W. A. Milne, P. Goldman, J. L. Holtzman, *J. Biol. Chem.* **243**, 5374 (1968).
53. S. Kobayashi, S. Kuno, N. Itada, O. Hayaishi, S. Kozuka, S. Oae, *Biochem. Biophys. Res. Commun.* **16**, 556 (1964).
54. J. W. Daly, G. Guroff, S. Udenfriend, B. Witkop, *Biochem. Pharmacol.* **17**, 31 (1968); J. Renson, *Fed. Proc.* **23**, 325 (1964).
55. G. Guroff, K. Kondo, J. Daly, *Biochem. Biophys. Res. Commun.* **25**, 622 (1966); G. Guroff, J. W. Daly, D. M. Jerina, J. Renson, B. Witkop, S. Udenfriend, *Science* **157**, 1524 (1967).
56. R. A. Peters, L. R. Murray, M. Shorthouse, *Biochem. J.* **95**, 724 (1965); R. A. Peters, M. Shorthouse, P. F. V. Ward, *Life Sci.* **4**, 749 (1965).

Genetic Load and Its Varieties

The term "genetic load" has been used to describe various situations, some harmful, some beneficial.

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In the aftermath of World War II, when the widespread dispersal of artificially produced radioactivity was a cause of serious concern, Muller discussed the problems of past and future damage to the genetic material of man in a paper entitled "Our load of mutations" (*1*). The term *genetic load* has been used since then to mean the abnormalities, deformities, and deaths produced in every generation by defective genetic material carried in the gene pool of man. Since the results of a mutation may not become evident until a number of generations later, it was feared at that time that radiation-induced mutations might reach dangerously high levels before the extent of the threat was appreciated.

The normal genetic load—the load that existed before the production of ionizing radiation by artificial means—consists of changes in the genetic material which occur at a very low but consistent rate and are referred to as "spontaneous" mutations, although numerous possible causes are known, including natural background radiation. It is sometimes stated that "all mutations are harmful." This is not strictly true: evolutionary progress has depended on mutations that were advantageous. But it is generally true, and two types of explanation are offered: (i) that in a complex and interdependent system such as the genetic configuration of a living organism, any change is more likely to disrupt function than to improve it, and (ii) that "good" mutations, when they appear, replace their precursors so rapidly that we rarely witness the process. In any case, the mutations of which we are most aware are those which result in impaired form or function. These tend, after appearing, to be eliminated as a result of shortened life span or lessened reproductive capacity of the individuals carrying the mutant genes. Under stable conditions, with the mutation rate and the intensity of selective elimination both constant, a particular mutant gene remains constant in numbers. Either an increase in mutation rate or a relaxation of selection will establish a new equilibrium at a higher level. (The effect of relaxed selection, resulting from medical alleviation of hereditary ailments, had been a cause of concern long before the possibility of increased mutation rates was envisaged, and was extensively discussed by Muller.) When an equilibrium has been established, the number of mutant genes eliminated per generation equals the number newly produced by mutation. However, if the equilibrium is disturbed, this is not immediately true; since many harmful mutants may remain in the gene pool for several or many generations before being eliminated, the full impact of genetic load will not be apparent until some time

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