The Problem of Drug Resistance in Cancer Chemotherapy*

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Several compounds are now in use which are often effective in inducing partial, or sometimes almost complete, remissions in certain types of neoplastic disease of man, particularly, but not exclusively, the acute leukemias of children. Among the especially useful drugs are certain analogs of folic acid, especially amethopterin ("Methotrexate"); the purine derivative, 6-mercaptopurine ("Purinethol"); and the so-called alkylating agent, busulfan ("Myleran"). Unfortunately, these compounds do not always cause remissions, even in those leukemias which differ in no discernible respect from those which respond. Furthermore, when remissions are induced it is a foregone conclusion that relapse will eventually take place, despite the continued administration of the originally effective drug. Of even less clinical utility than the three drugs referred to, at least in terms of duration of induced remissions when the compounds are used alone, are the glutamine antagonists, O-diazoacetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (DON), and certain steroids of the glucocorticoid group.

In cancer chemotherapy, therefore, we are presently faced with a clinically somewhat discouraging situation in which accurate predictions cannot be made concerning the initial effectiveness of a given drug in any single individual or concerning the duration of a remission if one is obtained. In fact, the only accurate prediction which can now be made is that, in almost any patient in whom a favorable response to a chemotherapeutic agent is obtained, there will eventually develop a fatal state of refractoriness which is usually referred to as drug resistance. The purpose of this paper is to consider this serious problem, to describe some of the present concepts concerning mechanisms which are involved, and to discuss the prospects, if any, of attaining chemotherapeutic results of greater significance, despite this problem. The present state of our knowledge, although the result of many relatively recent advances, certainly does not offer any profound solutions to this basic pharmacological problem of drug resistance which, in one way or another, has been investigated by many scientists during the last half-century.

In the field of bacteriology much knowledge has been accumulated concerning the origins of drug resistance. With microorganisms, resistance to a great variety of toxic agents can be developed, and in many cases this resistance can be attributed to the selection by the drug of mutant forms of the organism which perchance are relatively insensitive to the agent (44). In other words, a chemotherapeutic agent may markedly inhibit the reproductive capacity of the great majority of a population of cells, but those cells which are spontaneously relatively resistant serve as the progenitors of a new population of drug-resistant cells. In such cases, the drug acts as a selecting rather than as an inducing agent.

In the field of clinical bacterial chemotherapy the contribution of the host to the abolition of infection is often sufficient to prevent the emergence of drug-resistant forms, since drug resistance is usually present in only a relatively small proportion of the total population. Ordinarily, drug resistance probably becomes a serious clinical problem only in those cases in which the infection is caused by strains of microorganisms in which the population already contains an unusually large number of resistant cells. However, in the chemotherapy of neoplastic diseases, in which the contribution of the host tissues to the limitation of proliferation of the malignant cells usually appears to be quite small, the problem of the development of drug resistance is indeed a major one. In human cancer, in contrast to infectious diseases and to many of the mouse neoplasms which have been studied, we deal with neoplastic cells which arose from normal cells of the host and whose biochemical mechanisms appear to differ but little, often only quantitatively, from those of rapidly reproducing normal cells in the same organism. As a consequence, the most potent

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And drug-resistant lines of neoplastic cells of many species in vivo, and (e) other factors which may operate to affect the relative more rapid proliferation of the neoplastic cells than of those of normal tissues. Because of this, the susceptibility of the malignant population to an agent may appear to be greater, even though the actual toxicity of the agent for neoplastic cells may be quite similar to that for many normal cells.

An opportunity for selective toxicity may be afforded by the existence of independent pathways for the synthesis of biochemically essential compounds (for example, the endogenous biosynthesis by cells of purine or pyrimidine derivatives, versus the utilization of preformed substances of this type supplied in the extracellular fluids); thus some tumor cells may be less versatile in the use of one of these independent pathways than are the normal tissues. Only too often, however, the biochemical versatility of the cancer cell appears to be equal to that of normal host tissues.

Other factors which may be involved to a major extent in determining the efficacy of each potential chemotherapeutic agent are (a) the rate, extent, and location of its metabolic inactivation, (b) its metabolic conversion to an active form, (c) its transportability, either before or after this conversion, to all cancer cells, often distributed widely throughout various tissues of the body, (d) the rate of elimination of the active agent from the body, and (e) other factors which may operate to a greater or lesser extent in special cases. These problems of metabolic alteration and physiological disposition of potential anti-cancer agents, which only recently seem to have been given much consideration, will occupy much of our attention in this article. In fact, at the present time, it is important to ask whether our knowledge of the development of refractoriness to a previously effective agent, as seen in clinical cancer chemotherapy, should be discussed solely or primarily in terms of the selection of drug-resistant mutants of the population of neoplastic cells, or whether other factors may also be of great significance?

Let it be stated at once that there is not the slightest doubt concerning the essential validity of the many studies carried out with drug-susceptible and drug-resistant lines of neoplastic cells of many varieties in mice of many strains. It is worthy of mention that drug-resistant lines of some mouse leukemia strains can now be developed (or selected) in vitro. Thus, in our laboratories, Dr. Glenn Fischer and his associates (18–16, 48) have progressively cultured two highly malignant strains of leukemia of mice: the P388 mast-cell leukemia (of Dunn and Potter) and the L5178 lymphoblastic leukemia (of Law). From the latter strain have been obtained several new genetically stable lines which will grow in extremely high concentrations of amethopterin, a compound which is very inhibitory to the growth of the parent strain both in vivo and in vitro. For example, with a parent amethopterin-sensitive strain (L5178-Y; initially derived from a single leukemic cell), there have been obtained, by growth of the cells in progressively higher concentrations of amethopterin in the culture medium, resistant strains which can reproduce when cultured in the presence of this agent in concentrations up to 100,000 times that which was inhibitory initially. That such lines are derived from stable mutant cells is indicated by the fact that prolonged periods of reproduction in the absence of drug, either in culture or in mice, rarely leads to acquisition of increased sensitivity to amethopterin. Possibly, this may be regarded as only a biochemical or genetic "stunt" with mammalian cells, analogous to that already accomplished many times with bacterial populations, since the magnitude of the drug resistance obtainable in the controlled environment of a culture in vitro appears to be very much greater than that attained when chemotherapeutic agents are used in vivo. However, as will be mentioned later, it is hoped that this new technic will make possible certain studies of mechanisms which hitherto could not readily be undertaken with mammalian cells. Clearly, the host plays no necessary role in the selection of drug-resistant cancer cells, and there are no obvious differences in the conditions required for such selections when mammalian rather than bacterial cells are grown in semi-synthetic media.

It is pertinent to comment here on some of the limited information which bears on the question of the degree of drug resistance which cancer cells may acquire in vivo. Thus, in 1954 and subsequently, the relative capacity of amethopterin to inhibit, in vitro, the sensitive enzyme system which is the site of action of the drug (i.e., the conversion of pteroylglutamic acid [PGA] to functionally active forms [5, 39, 40]), was examined by Nichol (37, 38, 41) in these laboratories. In much of this work, cells of amethopterin-sensitive and -resistant strains of Line I leukemia (of C58...
mice), which had been developed in vivo by Burchenal, were used. The difference in susceptibility of the enzyme system to the inhibitory action of the drug with the two types of intact cells was quite small: a fourfold higher concentration of amethopterin inhibited the enzymatic transformation of pteroylglutamic acid by the resistant cells, as compared with that required for inhibition of the sensitive cells. However, it must be appreciated that this degree of alteration in biochemical susceptibility to amethopterin is quite sufficient to account for the resistance to the drug which had been observed in vivo. Amethopterin is usually administered in doses approaching those which are maximally tolerated, and thus the size of the doses used usually cannot be doubled, much less increased fourfold, without incurring intolerable host toxicity.

In the experiments just described the drug-resistant cells of Line I leukemia were obtained by their consecutive passage through amethopterin-treated mice until the drug, in the doses used, no longer was able to increase the survival time of the animals. However, with human leukemia the situation is quite different. There we not only encounter a spontaneous rather than a transplanted neoplasia, but also we deal with only one person, rather than consecutive individuals through whom the leukemia is passed, as is the case with the selection of drug-resistant strains in mice. Although it is generally accepted that the progressive treatment of initially drug-sensitive leukemic human subjects with amethopterin or other agents often leads to the gradual conversion of the population of leukemia cells to one which is drug-resistant, it is most difficult to obtain definitive data to prove that such is the case. This is because the presently available experimental methods do not lend themselves readily to appropriate quantitative studies of leukemia cells from a human subject, both before and after the development of a state of refractoriness to a given chemotherapeutic agent. If and when the very encouraging results of Fischer on the culture in vitro of certain mouse leukemias can be extended reliably to human leukemias, definitive biochemical data on the role of genetically determined drug resistance should be readily obtainable in man.

Since it is presumably somewhat analogous to the situation in human beings, let us consider briefly what happens in mice with an amethopterin-sensitive leukemia (L1210), when initially treated with this drug. Some years ago Law found (31) that, even though the survival time of such treated animals was approximately doubled, as compared with that of untreated controls, leukemia cells obtained from the treated mice just before death, and inoculated into new animals, were still quite susceptible to amethopterin. Similar results have been obtained in our laboratories by Jaffe and Sweedler.1 In their studies, the effect of amethopterin on the growth of L1210 leukemia cells in the peritoneal cavity of mice was determined (Chart 1). In the untreated controls the ascites cell counts, following inoculations of each mouse with about 3,000,000 amethopterin-sensitive cells, rose rapidly within 4 days to about 700,000,000, and the mice died in only 6 days

![Chart 1](chart1.png)

Chart 1.—The effect of alternate-day treatment with amethopterin on the growth of L1210 (ascites form) (see text for details).

with total lymphoblast counts averaging about 800,000,000. In the animals given injections of the customary doses of amethopterin (i.e., 3 mg/kg every 48 hours), beginning 2 days after inoculation with the lymphoblasts, the ascitic cell counts fell sharply after each injection. Although the trend of the counts was steadily upward in the treated animals, the ascitic cell count on the 10th day, about 290,000,000, still dropped sharply after the fifth and last dose of amethopterin to about 94,000,000 on the 11th day; nevertheless, death occurred in 12 days, when the final ascitic cell count probably was not more than about 290,000,000. Questions that might be asked are (a) Why did the treated mice die? and (b) Was the cell population of the treated, dying mice appreciably drug-resistant? The saw-toothed shape of the semilogarithmic response curve shown in Chart 1

1 J. J. Jaffe, and A. S. Sweedler, unpublished observations.
demonstrates that the leukemic population was largely composed of drug-sensitive cells; in addition, it was proved that the terminal ascitic population was still relatively drug-sensitive. Thus, lymphoblasts from the peritoneal cavity of a dying, amethopterin-treated animal were used for inoculation into new control mice, as well as mice then treated with the same doses of amethopterin as before: the control animals died in the usual 6-day period, while the survival of the treated mice was extended to 10 days rather than to 12 days as in the first phase of the experiment. Only with four successive passages through drug-treated mice was a strain of cells obtained which caused a fatal leukemia unaffected by these doses of amethopterin.

Why, then, did the first group of amethopterin-treated mice die? Although the answer to this question cannot be stated in absolute terms, some fairly reasonable guesses can be made. Since the drug-sensitivity of the population of neoplastic cells in the peritoneal cavity of the mice had not been greatly altered, and because the cell count in this space could not have exceeded about one-fourth that of the untreated mice, death presumably resulted from the effects of leukemia cells growing elsewhere than in the peritoneal cavity.

Of particular importance, in terms of the potential lethality of their infiltration, are critical areas of the bone marrow and the central nervous system, although the damaging effects of invasion of the liver, kidneys, and possibly other tissues cannot be disposed of easily. That the central nervous system may be of particular importance, however, is suggested by recent studies (~32, 35, 36, 53, 54). Thus, it has been shown that amethopterin crosses the blood-brain barrier to an extremely limited extent, a circumstance which could explain, at least in some cases, the ultimate demise of both mice and human beings invaded by amethopterin-sensitive neoplastic cells. In fact, infiltration of the central nervous system with lymphoblasts is histologically demonstrable in over 60 per cent of human patients who have died with acute leukemia, while about 80 per cent show either hemorrhages or infiltration at autopsy (34); neurological manifestations of such invasion have been estimated as occurring in approximately one-half of patients with acute leukemia (55). Even if diagnosis of acute leukemia is ever made prior to the invasion of the tissues of the central nervous system, therapy with tolerated doses of amethopterin probably cannot prevent a slow but progressive increase in the number of the cells, as was indicated by the data for mice, depicted in Chart 1. Accordingly, this circumstance would afford an opportunity for eventual invasion of areas to which amethopterin is not sufficiently transported for it to accomplish there very significant inhibition of cellular proliferation, and, therefore, damage to vital tissues such as those of the central nervous system eventually may occur. In addition, growth of cells in such areas provides niduses for the continual re-entry of leukemia cells into the circulation.

Bearing on this problem very significantly are the important studies of Li, Hertz, and their colleagues at the National Cancer Institute (22, 35, 36), which demonstrate that what appear to be complete remissions often can be obtained in women with the extremely malignant disease, choriocarcinoma, when the patients are given intensive parenteral therapy with amethopterin. However, relapses are now appearing in many of their patients, with growth of cancer cells in the lungs as well as in (in some cases) the central nervous system. Growth of cells in the former location is attributable to the selection of a drug-resistant strain, but growth within the cranium has contributed to vigorous investigation of the many problems encountered when drugs such as amethopterin are injected directly and frequently into the fluids bathing the tissues of the central nervous system of man. Also, it has contributed emphasis to the need to investigate, far more thoroughly than has been the case in the past, the distribution of this and other anti-cancer agents to the various organs and tissues of the body, particularly their capacity to cross the blood-brain barrier. The point which obviously is to be made at this time is that the relapses in this initially amethopterin-susceptible form of cancer may be due not solely to the emergence of drug-resistant cells, but also to the failure to attain adequate concentrations of the drug in all tissues infiltrated by initially drug-susceptible members of the population of cancer cells. It should be mentioned that, in at least one of the most responsive choriocarcinoma cases of Hertz et al. (32), manifestations of invasion of the central nervous system disappeared following intramuscular therapy with amethopterin. Consequently, in this apparently very sensitive neoplasm the transport of the drug across the "barrier" must have been sufficient to affect markedly the neoplastic cells within the cranial cavity. However, as will be developed in the following paragraphs, the capacity for amethopterin to cross the blood-brain barrier is, clearly, extremely low.

Bearing importantly on this concept is the work of Whiteside, Burchenal, and their associates (53, 54) at Memorial Hospital in New York. These
workers have described remarkable improvement in the manifestations of neurological involvement during the course of acute leukemia, about 1 week following the intrathecal administration of amethopterin in doses of from 0.1 to 0.5 mg/kg. Since two of the patients studied were considered to be refractory to the drug when given orally, it may be assumed that the cells responsible for the neurological disorder were resistant to the concentrations of amethopterin attained by the oral route in the blood and other tissues, including those of the central nervous system. Thus, in a child of 10 years, 0.33 mg. of amethopterin/kg orally gave a peak serum level after 30 minutes of 900 mg/ml (but this fell precipitously within 3 hours) and a peak level in the cerebrospinal fluid at 3 hours of 15 mg/ml. On the other hand, intrathecal administration of 0.1 mg/kg gave a level of amethopterin in the cerebrospinal fluid of 5,000 mg/ml at 1 hour and 475 mg/ml at 18 hours after the injection. With 0.5 mg of amethopterin per kg. given intrathecally, the cerebrospinal fluid level was 430 mg/ml, even 48 hours following the injection. Although these very high concentrations of drug in the cerebrospinal fluid do not necessarily indicate the concentrations within the brain parenchyma, the probability is strong that the attainment of neurologic manifestations is attributable to high concentrations of drug at sites of lymphoblast growth and to the resultant cytolysis, within the nerve tissues, of neoplastic cells which were quite resistant to concentrations attainable by transport across the blood-brain barrier.

It is of considerable interest that intrathecally administered amethopterin is remarkably free of toxic effects upon the central nervous system. In fact, the limitation on dosage by this route appears to be the rate at which the drug leaves the cerebrospinal fluid to enter the systemic circulation, thus to affect such sensitive tissues as the bone marrow and the mucosa of the alimentary tract. This circumstance is not too surprising if it is considered that the inability of nerve cells to reproduce may free them of the necessity to make deoxyribonucleic acid, the formation of certain precursors of which (in particular, thymidyl acid) is dependent upon coenzymes derived from folic acid (the formation of which is blocked by amethopterin); other metabolically utilizable compounds (e.g., glycine, serine, adenosine, etc.), related to the products of other reactions in which such coenzymes are involved, may well be able to cross the blood-brain barrier.

Perhaps it should be emphasized at this time that intrathecal therapy after the brain is very heavily invaded with lymphoblastic cells cannot be expected, as Whiteside, Burchenal, and associates have observed, to result in other than temporary cerebral improvement. In fact, on the basis of studies in mouse leukemia, it is remarkable that even advanced systemic leukemia (much less than of the central nervous system) responds to amethopterin when therapy is initiated relatively late in the course of the disease. Thus, Skipper and his associates have demonstrated a definite relationship, in mouse leukemia, between the effectiveness of amethopterin and the size of the neoplastic population at the time when therapy is initiated. With the use of an unequivocal bioassay for the presence of significant numbers of cancer cells, various tissues of mice were examined at intervals following the intraperitoneal injection of leukemia cells, L4946, in known numbers. Their data show that the neoplastic cells enter certain tissues very rapidly, e.g., liver, lymph nodes, and lungs; unfortunately, from the present point of view, assays of the bone marrow and, particularly, of the brain were not described. However, it was shown that the effectiveness of amethopterin as a "curative" agent against this and also L1210 leukemia in mice is inversely related to the number of cells present in the body at the time when therapy is initiated. Thus, inoculation with 100 L1210 leukemia cells caused the death of eight of ten untreated mice but was not lethal in any mice in which therapy with amethopterin was initiated 24 hours after the inoculation. Intraperitoneal inoculations with 10,000 leukemia cells, although fatal to all untreated animals, permitted subcutaneous injections of amethopterin (1 mg/kg/day) to abolish the early neoplasia in seven of ten animals. Even with inocula of 1,000,000 L1210 cells, amethopterin completely obliterated the neoplastic process in one half the animals, if subcutaneous therapy (1 mg/kg/day) was initiated 24 hours after the inoculation. It should be emphasized, however, that initiation of therapy 48 hours after inoculation undoubtedly would have given very much less favorable results with each of the three situations.

These findings are mentioned not only because of their very important clinical implications with respect to the potentially greater efficacy of chemotherapeutic agents prior to the time when a neoplastic process has become far advanced and various critical tissues have been invaded, but also because of their bearing on the problem of drug resistance. Thus, it might be thought that the treated animals which died did so primarily because of the occurrence of a barely sufficient num-
ber of drug-resistant cells to lead ultimately to death of the treated animals. However, the previously described experiments of Jaffe and Sweedler, with the same variety of mouse leukemia (L1210), render this possibility quite unlikely. To this reviewer it seems much more reasonable to propose that the animals which died despite treatment did so because of the distribution of a sufficient number of cells to, and their rapid proliferation in, sensitive tissues (e.g., the brain) to which the antimetabolite is poorly transported. Experiments designed to test this hypothesis are now in progress.

Surely, it is strongly indicated for intrathecal administration of amethopterin (and, when adequately investigated, of other drugs) to be carried out initially, rather than as a final measure of desperation. But even more strongly indicated is the need for logical attempts to modify existing chemotherapeutic agents in such manner as to promote their rapid transport not only across the blood-brain barrier, but also across those "barriers" presumably lying between blood and other specialized tissues, such as those of nerve trunks, adipose depots, fibrous tissues, etc. Such attempts at chemical modification can now be made on the basis of sound principles (for a review of the physico-chemical factors involved in physiological transport, see Brodie and Hogben [9]). In these laboratories studies are now in progress which are directed toward the possible development of "anti-folic agents" which might more readily cross the various "barriers" than is possible with compounds resembling amethopterin in structure, which are highly ionized at physiological pH levels.

The remarks made so far have been primarily for the purpose of calling attention to some of the problems of drug refractoriness which do not relate to the now much discussed areas of biochemical genetics and the chemical mechanisms by which certain members of a population of cells may escape the noxious effects of an otherwise potent cell poison. Certainly, this discussion is not meant to minimize the clinical importance of the selection of drug-resistant mutants or the significance of the fascinating genetic and biochemical problems afforded by genetically determined drug-resistant strains of neoplastic cells. In those leukemia patients who respond initially to chemotherapy but eventually relapse during the continued administration of the drug in maximally tolerated doses for several weeks or months, with a progressive increase in the invasion of the bone marrow and peripheral blood by neoplastic cells, a state of drug refractoriness clearly has developed. Although this refractoriness may be influenced by the so-called "population-effect" (i.e., the phenomenon already described in mice in which chemotherapeutic agents exert less and less effect as the size of the population of leukemia cells gradually rises [47]), it is reasonable to believe that a gradual selection of drug-resistant cells, which have served as the progenitors of all or a part of the terminal population in certain tissues, is operative. Accordingly, if genetically based drug resistance is a major factor in the drug-refractory clinical situation with amethopterin and 6-mercaptopurine, as it certainly appears to be with such drugs as azaserine, to which patients become refractory very rapidly, what can we learn from some of the more pertinent recent findings made with bacteria and with neoplasms of mice?

Davis and Maas (11) have discussed the different possible mechanisms of resistance based on biochemical concepts, and these are listed as follows:

1. decreased penetration of the drug
2. increased destruction of the drug (or decreased conversion of an inactive to an active compound)
3. increased concentration of a metabolite antagonizing the drug
4. increased concentration of an enzyme utilizing this metabolite
5. decreased quantitative requirement for a product of the metabolite
6. alternative metabolic pathway by-passing the metabolite
7. enzyme with decreased relative affinity for the drug compared with the metabolite.

Theoretically, any of these mechanisms could be encountered as a result of mutational changes which have resulted in specific biochemical lesions in the structure or function of a gene. With the template theory in mind, the gene-controlled synthesis of an enzyme is regarded as disturbed by a mutation; as a result, there is either an essential loss of a normal chemical reaction or an alteration in the nature and function of an enzyme critically concerned with the catalysis of such a reaction [23].

From the standpoint of an opportunity being afforded for the possible exploitation of drug resistance to the advantage of chemotherapy, it would appear preferable to have drug resistance accounted for by the complete loss of the activity of an enzyme—for this could reflect a truly qualitative, rather than a quantitative, difference between the drug-selected cancer cells and the normal cells from which the original neoplastic cell arose. It is almost a cliché today to point out
that the development of a really rational basis for guiding the design of potent, really selective chemotherapeutic agents demands the disclosure of such qualitative differences between normal and neoplastic cells (46). Indeed, in theory, it might be possible to use certain noncurative chemotherapeutic agents deliberately to convert a neoplastic population to a drug-resistant one, thus possibly to obtain a qualitative difference between the cancer cells and the normal cells of the organism, which might be exploited in a different way. Whether, in fact, such an ideal situation ever will be attainable is, of course, quite impossible to say at present. It is difficult enough even in mice used consecutively as hosts of leukemia cells, and given maximally tolerated doses of drugs, to attain other than quantitative changes in the degree of sensitivity to chemotherapeutic agents, at least in those cases so far studied.

The profoundly drug-resistant cell, when obtained under the much more nearly ideal conditions of environmental control which are possible with bacteria grown in vitro, and apparently also when neoplastic cells are grown in culture, often will have displayed a stepwise increase in degree of resistance (as with penicillin and amethopterin), presumably owing to sequential mutational changes. On the other hand, a one-step development of marked resistance (as with streptomycin and azaserine) may be seen. Which, or what proportion, of these changes is truly of a qualitative rather than a quantitative nature is not yet clear.

It may be asked how a mutational change can lead to a quantitative alteration in enzyme activity, rather than to a loss of the enzyme? Horowitz has pointed out in a provocative review of chemical concepts of genetic phenomena (33) that, as a result of qualitative changes in enzyme structure, it is theoretically possible to obtain mutations that result in quantitative changes in enzyme activity. Indeed, he has commented on four examples of mutation-induced alterations in physico-chemical properties of soluble proteins; see Table 1.

Although these examples of alteration in the properties of proteins, without loss of their main function, obviously have been presented only to illustrate a principle, it is indeed an important one, and it has been given appropriate recognition by Davis and Maas (see point 7 of Davis and Maas [11]). Thus, genetically modified enzyme proteins may be encountered in which the affinity of an enzyme for its normal substrate may be altered to a less marked degree than for a compound which, for the original enzyme, was a potent inhibitor. Clearly, such a circumstance could account for drug resistance, and with such a mechanism in operation means of circumvention have not been visualized.

Rather than to discuss examples, wherever they are known to exist, of each of the possible mechanisms of resistance which have been presented by Davis and Maas, it would seem desirable now to discuss particularly the phenomenon referred to parenthetically by those authors (see point 2 of Davis and Maas [11]), i.e., "decreased conversion of an inactive to an active compound."

Especially pertinent to this mechanism, albeit carried out with bacteria, is the work of Handschumacher in our laboratories on the mechanism of resistance of Streptococcus faecalis to 6-azauracil (18). His observations indicated that the genetically stable, drug-resistant strain represented a selection by the analog of those cells of the population which fortuitously lacked the capacity ef-

### Table 1

Mutation-Induced Qualitative Alterations of Proteins
(from Horowitz [23])

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<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Property</th>
<th>Authors</th>
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<tbody>
<tr>
<td>Hemoglobin</td>
<td>Man</td>
<td>Electrophoretic mobility</td>
<td>Pauling et al.</td>
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<tr>
<td>β-Lactoglobulin</td>
<td>Cattle</td>
<td>Electrophoretic mobility</td>
<td>Aschaffenburg and Drewry Maas and Davis</td>
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<tr>
<td>Pantothenate-synthesizing enzyme</td>
<td>E. coli</td>
<td>Thermostability</td>
<td>Davis</td>
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<tr>
<td>Tyrosinase</td>
<td>Neurospora</td>
<td>Thermostability</td>
<td>Horowitz and Fling</td>
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This finding suggested the possibility that the ribonucleoside of azauracil (azauridine) could be utilized by and would inhibit the growth of the mutant which is resistant to azauracil. Indeed, this proved to be the case, and a deliberate biochemical circumvention of drug resistance was accomplished thereby, perhaps for the first time. It can be assumed that these mutant cells of *Streptococcus faecalis* are those which are grossly deficient in an enzyme normally concerned with the utilization of uracil through its conversion to uridine and thus to uridine-5'-phosphate (uridylic acid). Since the pathway for utilization of uracil is defective in the mutants, no opportunity for utilization of the analog is afforded and the mutants survive, whereas those capable of utilizing either uracil or azauracil are eliminated. The wild strain of the organism also possesses occasional cells which are defective in the utilization of uridine, and these...
survive in the presence of azauridine. Although not yet established, it is reasonable to suggest that these cells, resistant to azauridine, are deficient in the kinase normally concerned with the conversion of uridine to uridylic acid and lack, therefore, the capacity to perform a lethal synthesis of uridylic acid.

It is now clear, not only from studies with bacteria and yeast, but also with mammalian cells of both normal and neoplastic varieties (19, 20), that the site of action of metabolically formed azauridylic acid is the enzyme termed orotidylic acid decarboxylase, which forms uridine-5'-phosphate by decarboxylating orotidine-5'-phosphate. The formation of fraudulent nucleic acids does not appear to be an important factor in the mechanism of action of azauridylic acid; in fact, the mono- and poly-phosphate esters of azauridine do not appear to interfere to any significant degree with the metabolism of uridine and its several important phosphorylated derivatives (20). It should be emphasized, however, that inhibition of orotidylic acid decarboxylase is a potentially important locus of inhibition, since it leads to a block in the utilization of orotic acid, the first product of the sole or main pathway of biosynthesis de novo of all pyrimidines of cells; in the same manner, utilization of exogenous orotic acid, e.g., from dietary sources, can be blocked by adequate levels of azauridylic acid.

The availability of this biochemical tool may afford another useful possibility, namely, the assessment of the relative importance in different types of cells of the acquisition of uridine derivatives by synthesis de novo, as compared with utilization of preformed pyrimidines. In this connection it is of considerable interest that, in dogs, azauridine profoundly inhibits the formation of both lymphocytes and granulocytes and, to a lesser extent, platelets, without causing significant injury to the intestinal mucosa, while in mice the drug inhibits the growth of several transplanted neoplasms at doses which do not cause important manifestations of toxicity (26). Since it is well known that the intestinal mucosal cells can utilize either free uracil or uridine (10, 21, 27), these findings would suggest, therefore, that the utilization of the "preformed pathway" is the means by which the intestine can survive the presumed blockade of its biosynthesis of pyrimidines de novo. On the other hand, it may be suggested that the lymph nodes and bone marrow, at least of dogs, and some transplanted neoplasms, at least of mice, have a relatively limited capacity for the utilization of preformed pyrimidines, other than perhaps orotic acid. Thus, even if azauridine should prove to have no value in the treatment of neoplastic disease in man, it will have afforded encouragement concerning the development of other agents, the selective toxicity of which could enable them more markedly to injure one type of reproducing cell than another.

This new agent has been discussed in some detail, not so much because of the current interest in it in our laboratories, but because it affords an opportunity for discussing briefly what may be termed conditioned selectivity. Thus, cells which have been selected by azauridine, because of the fortuitous lack in them of an adequate pathway for the utilization of uracil and uridine, and therefore of their analogs, may experience difficulty in obtaining their essential complements of pyrimidine derivatives from preformed materials in the fluids surrounding the cells. In other words, such drug-resistant cells may be forced to depend upon the biosynthesis of pyrimidines de novo. If this should prove to be a prominent mechanism of the resistance of neoplastic cells to azauridine, as has been found to be the case with Streptococcus faecalis, and if the metabolic defect is quantitatively sufficiently marked, the manufacture of pyrimidines de novo should be obligatory in an azauridine-resistant population of cancer cells. In such a situation, a major difference between the population of neoplastic cells and the normal cells of the organism would have been developed, and the stage would thus be set for a different type of potentially very selective inhibition of the biosynthesis of pyrimidine-containing compounds from small molecular precursors (e.g., via aspartic acid, carbamylaspartic acid, dihydro-otic acid, 4-otic acid, and orotidylic acid). With such a development, the search for effective inhibitors of the formation or utilization of such metabolic intermediates would be all the more indicated.

Evidence for the deletion of other enzymes concerned with the formation of active metabolites of anti-cancer agents, as a mechanism of drug resistance, has been obtained recently. Thus, Brockman and his associates (5, 7, 8) have recently studied the mechanism of resistance of Streptococcus faecalis to 8-azaguanine and have found that the circumstances are similar to those described for 6-azauracil (18), since the azaguanine-resistant organisms, unlike the sensitive parent strain, are grossly deficient in their capacity to form the ribonucleotide (azaguanilic acid) from azaguanine, and thus to incorporate the latter into the nucleic acids of the cells. Although the parent strain can utilize for growth either adenine, guanine, hypoxanthine, or xanthine, this resistant strain grows only on xanthine. It is apparently de-
sufficient in an enzyme concerned with the ribonucleotidation of guanine and hypoxanthine; adenine is converted to the mono-, di- and triphosphates of adenosine (i.e., AMP, ADP, and ATP), but, despite this, it is a poor precursor of inosinic and guanylic acids (2). Preliminary studies with cell-free extracts support these statements (5). With this azaguanine-resistant mutant, the use of ribonucleosides offers no advantage, for the metabolic pathway does not appear to involve the intermediate formation of the purine ribonucleosides, but utilizes phosphoribosylpyrophosphate (PRPP) for the direct ribonucleotidation of the purines. On the other hand, this mutant can utilize xanthine, and, interestingly enough, is inhibited by its analog, 8-azaxanthine, which it converts into the inhibitory ribonucleotide, 8-azaguanylic acid, presumably by way of 8-azaxantholic acid. This also may be regarded as another example in bacterial metabolism of a biochemical circumvention of drug resistance.

This azaguanine-resistant strain closely resembles a strain selected for resistance to 6-mercaptopurine (6-MP) (25); whereas the parent strain forms the ribonucleotide, this resistant strain (a) did not do this, (b) was cross-resistant with azaguanine, and (c) was inhibited by azaxanthine (4). However, other strains of *S. faecalis* selected for resistance to 6-MP have shown interesting differences from the one just described (25). Thus, one resistant strain (SF/MP) grew only on xanthine, but cell-free extracts were shown (5) to convert adenine to adenylic acid; in this case a metabolic defect in the conversion of the adenylic acid to inosinic acid has been postulated, since the formation of guanylic acid from inosinic acid (when synthesized de novo) appears to be normal. Another 6-MP-resistant strain (SF/MPcc) retained some of its capacity to convert hypoxanthine and guanine to ribonucleotides, but did not convert 6-MP to a ribonucleotide; it is suspected that in this situation there may indeed be an altered affinity of the enzyme for the analog (5).

Resistance to azaguanine in *S. faecalis* can lead not only to the loss of the capacity to utilize for growth the three purines: adenine, guanine, and hypoxanthine (SF/AZAG), but also to loss of capacity to grow on xanthine (SF/8-Aza) (5). When this occurs the organism is forced to depend exclusively on the biosynthesis de novo of purines and, therefore, should be extremely sensitive to effective inhibitors of this pathway, e.g., amethopterin. However, this theoretical expectation is not always realized. Thus, Hutchison, who studied such a mutant of *S. faecalis*, found that both the requirement for folic acid and the sensitivity to amethopterin were somewhat diminished, rather than increased, as might have been expected (25). It is conceivable that this strain of cells represents a selection of mutants with an unusually high capacity to synthesize purines de novo and thus a reduced requirement for folic acid; however, to explain the unexpectedly reduced effectiveness of amethopterin is difficult, and one is tempted to fall back upon the concept of a fortuitously diminished affinity of the folic acid reductase system for amethopterin, an explanation not yet subjected to experimental verification.

Although considerable increases in susceptibility to amethopterin have been demonstrated by both Law (28-32) and Skipper and associates (48, 49) in studies of mouse leukemias, particularly L1210, selected for resistance to azaguanine or 6-MP, in clinical practice there have been no reports to indicate that the level of resistance to 6-MP attained in acute leukemias has been of sufficient degree demonstrably to "sensitize" to therapy with amethopterin. Here again one must assume that the degree of resistance to the purine analogs which is attained *in vivo* is not sufficient to close off the pathway of utilization of the naturally occurring purines, or that there has been a selection of cells with a high capacity for synthesis de novo and a low affinity for the folic acid antagonist.

It should not be concluded from this brief description of various studies of mechanism of resistance to analogs of purines that the sites of action of azaguanine and 6-MP are identical or, in fact, that these agents necessarily affect only the utilization of preformed purines. It is noteworthy that the observations of several groups indicate that 6-MP and 6-thioguanine (chemically a closely related anti-cancer agent) interfere as well with the capacity of cells to synthesize purines de novo. Thus, in 1954, Skipper reported (45) that, in mice bearing Sarcoma 180 or Adenocarcinoma 755, 6-MP inhibited the incorporation of the carbon of formate-C14 into the nucleic acids of both tumors and intestine but failed to inhibit the utilization of adenine for this purpose. Although the incorporation of hypoxanthine into intestinal polyribonucleotides of normal mice was inhibited by 6-MP (45), the very marked interference with the biosynthesis of purines from small molecular precursors does not seem to be generally appreciated, nor has an adequate explanation of this interference been offered, although a mechanism resembling that of the "feed-back" principle may be entertained.

The studies of Brockman and his associates (4-8) of the mechanisms of resistance to azaguanine
and 6-MP have considerable importance from the standpoint of our rapidly developing knowledge of the mechanisms of drug resistance, even though they have been concerned primarily with microorganisms rather than with cancer cells and, in part, with a drug (azaguanine) which presently appears to have little or no prospect for clinical utility because of unfortunate side-effects. Already these studies have their counterparts in work with neoplastic cells. In fact, Brockman and his associates (6) have found that L1210 lymphomas selected by Law for resistance to azaguanine, and grown on one side of mice carrying azaguanine-sensitive tumors on the other, take up almost none of the C14-labeled anti-cancer agent in the resistant tumors, while the nucleic acids of the drug-sensitive tumor cells become markedly labeled with the analog. Resistance to this agent in cancer cells, and perhaps to 6-MP as well, may prove to be attributable, at least in part, to a selection of cells deficient in an enzyme concerned with the conversion of the analog to the corresponding ribonucleotide, a circumstance which might reflect, therefore, a corresponding deficiency in the assimilation of preformed purines. In closely similar studies, Sartorelli and associates have observed (49) that Ehrlich ascites carcinoma cells resistant to 6-thioguanine do not convert it to thioguanine in vivo as do the cells of the parent sensitive line. Although this difference was attributed by these workers to a greater capacity of the resistant cells to break down 6-thioguanine, this might prove to be a secondary manifestation of an altered capacity of the cells to form the ribonucleotide of the analog.

Another area of the problem of drug resistance in cancer chemotherapy which deserves special comment is that of combination therapy. Hailed as a means of avoiding the selection of resistant mutants and of obtaining much greater inhibitory effects on tumors, the results, in general, have been quite disappointing. Let us examine briefly some of the possible reasons for this. In the first place, there is nothing obviously wrong with the basic concept. If, for example, with each of two drugs, a resistant mutant may be expected to appear only once among $10^{10}$ cells, then a combination of the two drugs should reduce the probability of the appearance of a doubly resistant mutant to once in $10^{30} \cdot 10^{12}$ cells, and, in addition, an excellent opportunity for chemotherapeutic potentiation might well be afforded. However, the potentiation of activity for abnormal cells is usually accompanied by a comparable increase in the toxicity of such combinations for normal tissues of the host, and thus a significant therapeutic advantage is but rarely attained. Accordingly, the use of combinations of drugs usually has not been followed by significantly more favorable clinical results than those obtained with one or the other member of the pair; perhaps the most notable of the combinations thus far studied is that of 6-MP and azaserine. Since both of these agents inhibit purine metabolism, but at different loci, an excellent opportunity is afforded for potentiation and for diminishing the frequency of emergence of drug-resistant cells; clinical studies appear to have justified the use of the combination. Studies in tumor-bearing animals have demonstrated the rather marked potentiation of the action of azaserine by 6-MP or by thioguanine (51). Even in a situation in which the cell line employed (a strain of L5178) had been selected (in vivo) for relative resistance to 6-MP, the marked inhibitory activity of azaserine was clearly potentiated by 6-MP. In fact, with the combination, growth of the lymphomas was prevented completely; but, even under these most favorable conditions, cures were not obtained.\footnote{J. J. Jaffe, unpublished observations.}

Several years ago it was reported by Skipper and his associates (50) that a combination of 6-MP and amethopterin only very moderately extends the survival time of mice with L1210 leukemia, as compared with that resulting from the use of either drug alone. The findings obtained in a typical experiment are summarized in Table 2. It may be assumed without too great hazard that the ultimate demise of the animals given the combination was not due to the escape of drug-resistant mutants, but is to be accounted for by the incomplete suppression of lymphoblast proliferation by the combination of agents. With

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Treatment & Amethopterin (mg/kg/48 hr) & 6-Mercaptopurine (mg/kg/48 hr) & Average life span (days) \\
\hline
None & & & 10.6 \\
Amethopterin & 3 & & 28.3 \\
& 1.5 & & 18.9 \\
6-Mercaptopurine & & 40 & 14.4 \\
& & 20 & 12.6 \\
Amethopterin + & & & \\
6-Mercaptopurine & 1.5 & 20 & 27.2 \\
\hline
\end{tabular}
\caption{Effect of Amethopterin and 6-Mercaptopurine, Singly and in Combination, on Survival of Mice with L1210 Leukemia}
\end{table}
azaguanine and amethopterin in combination, the results with this same leukemia are much more impressive, while with L5178 the effect of the combination of 6-MP and amethopterin is far more striking than it is with L1210 (28-32). In fact, when 6-MP and amethopterin were used sequentially, rather than simultaneously, a goodly percentage of cures was obtained by Law1 with L5178, although not with other leukemia strains. This positive result cannot be accounted for by assuming that the initial use of 6-MP, for a period of only a few days, led to the selection of drug-resistant cells which were particularly dependent on syntheses of purines carried out de novo, and thereby highly susceptible to amethopterin. Possibly, 6-MP, although not capable of cure by itself, reduced the population of cancer cells to such a level that amethopterin then was able to cope with the remainder of the neoplastic population. Since these results were not obtained when the order of administration was reversed, i.e., when amethopterin was given first, the suggestion may be entertained that the initial use of 6-MP is successful because this agent crosses the blood-brain barrier sufficiently well (17) to prevent the proliferation of malignant cells in the brain. On the other hand, the primary use of an agent, such as amethopterin, which is very poorly transported across the blood-brain barrier, may permit the early establishment of niduses of neoplastic cells within the central nervous system. Investigation of this possibility is now in progress.

For those interested in obtaining much more detailed knowledge concerning many aspects of drug resistance in the chemotherapy of experimental leukemias than it has been possible to present in this brief discussion, the excellent review (1956) of Lloyd Law (31) is recommended.

In the remaining paragraphs the author will indulge, however insecure the supporting evidence may be, in some rather wild speculation concerning the possible experimental approaches to circumvention of the problems of drug resistance in cancer chemotherapy. For example, as has been suggested earlier in this article, could drug resistance be made really critically advantageous to the subsequent use of another potent agent? If a level of resistance to a true "anti-purine" could be obtained in such a manner that the capacity for the utilization of preformed purines would be essentially abolished, so that the altered population of neoplastic cells would become critically dependent on the intracellular synthesis of purines de novo, selective sensitivity to inhibitors of the latter pathway should be gained. Similarly, if a degree of resistance to such a drug as azauridine could be obtained which made the new population profoundly dependent on the intracellular synthesis of pyrimidines de novo, might we not be able to devise a means of exploiting such a situation? Is there a way in which the development of drug resistance of these specific types could be favored?

Although answers to these presently rhetorical questions have not yet been found, the lessons of modern bacteriology stand before us as a challenge. Thus, with certain types of microorganisms, the frequency of appearance of specifically drug-resistant cells in a population of normal cells can be greatly increased by the growth of such cells in the presence of deoxyribonucleic acid (DNA) extracted from drug-resistant organisms of the same species (1, 55, 56). Although such dramatic findings have not been obtained with mammalian cells, there is no known reason why this should not some day be possible. In fact, as has been mentioned previously, the availability of L5178 cells which can be grown in suspension in a medium which is now almost chemically defined (12-15), can be handled with the technics of classical bacteriology, and from which strains of cells tremendously resistant either to amethopterin or to azauridine have been selected, has made it feasible to attempt to transform the drug-sensitive lymphoblasts to drug-resistant cells by the appropriate use of DNA obtained from drug-resistant cells.

The possibility of attaining similar transformations in vitro is by no means too remote to consider, for the initial demonstration of transformation was actually carried out in vitro (Griffith [16]). In this now classical work of 1928, a relatively small number of living avirulent (R) pneumococci were injected into mice together with a large number of heat-killed, highly virulent Type III (S) pneumococcal cells. The animals frequently succumbed to infection, and pure cultures of Type III pneumococci could then be recovered from the blood. It was this work which served as the basis for the subsequent studies of Avery et al. (1) that initially related the transforming principle to DNA.

If an appropriate transforming DNA could be obtained from mammalian cells, which indeed would cause transformation of neoplastic cells to a desired drug-resistant state, or even resistant cells to a drug-susceptible state, it is not inconceivable that such DNA might some day be prepared in quantities. Recently, Kornberg and his associates (9, 33) have described directed syntheses of various types of DNA, using the DNA-synthesizing system obtained from extracts of E. coli. Although, as yet, none of the DNA-like materials obtained

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1 L. W. Law, personal communication.
has been capable of causing bacterial transformations, it is highly probable that success will eventually be attained. In any case, there is no reason to take a defeatist attitude concerning the possibilities which further investigations in these areas may disclose.

In conclusion, then, the fundamental purpose of this article has been to emphasize three points: one, that the magnitude of the problem of the selection of genetically stable, drug-resistant cells has not yet been clearly established in human cancer chemotherapy; two, that in the future much greater emphasis should be placed, than has been the case in the past, on the problems involved in insuring that anti-cancer agents are endowed with those physico-chemical features which are essential for the drugs to reach susceptible neoplastic cells in all tissues of the body; and, three, that however complicated the biochemical problems of genetically determined drug resistance may prove to be, the ingenuity of the human mind may be counted upon ultimately to find a way to circumvent the inimical machinations of mutations which result in resistance to otherwise effective chemotherapeutic agents.

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The Problem of Drug Resistance in Cancer Chemotherapy

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