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Introduction

An understanding of the biochemical strategy of the cancer cell is the via regia for the design of enzyme pattern-directed anticancer chemotherapy. The development and impact of novel approaches that provided us with paradigms for problem solving in these two central areas of cancer research will be emphasized in this presentation. I will underline the essence and substance of our conceptual and experimental advances towards an understanding of the biochemical strategy and the logic of gene expression in cancer cells and identify a shared enzymic and metabolic program in various animal and human neoplasms. With the pinpointing of the selective advantages that the altered gene expression confers on cancer cells, sensitive targets and ordering principles have emerged for the rational design of enzyme pattern-targeted chemotherapy (97, 98).

Biochemical Basis of Chemotherapy

All cancer cells are transformed. Thus, they have a biological commitment to continued replication which is manifested in a biochemical program of quantitative and qualitative imbalance. Cancer cells are also characterized by the capacity for a gradual escalation in expression of neoplastic properties, i.e., progression, an amplification of the biochemical imbalance.

Drug treatment should induce a metabolic perturbation in cancer cells that would selectively interfere with the biochemical program of the commitment to replication and result in the death of cancer cells. The host cells should adapt and recover from toxicity.

In the following, an account will be given of the main ideas and experimental observations that led to the formulation of the present integrated concept of the biochemical strategy of cancer cells.

In our laboratory, the introduction of three approaches provided instruments for discovery: (a) the molecular correlation concept; (b) the key enzyme concept; (c) the use of meaningful biological models and controls.

The Molecular Correlation Concept

In 1961, I first introduced the ideas of the molecular correlation concept as a theoretical and experimental method for discovering the pattern of biochemical imbalance and its linking with neoplastic transformation and progression (90). This approach provided precise, testable predictions for anticipated alterations in the enzymic and metabolic pattern of cancer cells (97, 98). Three main propositions were made:

1. The biochemical strategy of the genome in neoplasia can be identified by elucidating the pattern of gene expression as revealed in the activity, concentration, and isozyme program of key enzymes.
2. Activities of key enzymes and metabolic pathways and concentrations of strategic metabolites are stringently linked with neoplastic transformation and progression; those not stringently linked do not yield a pattern.
3. The linking of enzymic and metabolic imbalance with transformation and progression can be identified in a spectrum of tumors of graded malignancy, proliferative rate, and degree of differentiation.

Linking of Biochemical Strategy with Biological Behavior in Neoplasia. To identify the pattern of biochemical imbalance and its linking to neoplastic transformation and progression, three types of relationships were postulated and verified by the molecular correlation concept. The classification referred to the behavior of activities of key enzymes and overall metabolic pathways and of the concentrations of strategic metabolites (90, 97, 98, 109).

Progression-linked Alterations: Biochemical Expression of the Different Degrees of Malignancy. In Class 1 are the biochemical parameters that increase or decrease in a graded fashion in correlation with tumor malignancy. These are stringently linked markers of neoplastic progression.

The malignant program may be expressed in different degrees that range from mild through advanced to the full-blown pattern. This graded expression of the neoplastic program in the different tumor lines in a spectrum of neoplasms of the same cell type is termed "progression." Thus, the activities of key enzymes that are stringently linked with the biological behavior should be expressed in parallel with the degrees of tumor malignancy. Testing these predictions demonstrated that the activities and concentrations of key enzymes increased and those of the opposing enzymes decreased in concordance with the malignancy of the different tumor lines. With the increase in the expression of malignancy, i.e., proliferation rate, the enzymic and metabolic imbalance becomes more and more pronounced.

Transformation-linked Alterations: The Shared Programs of Cancer Cells. In Class 2 are parameters that increased or decreased in all hepatomas, irrespective of the degrees of neoplastic progression. These are ubiquitous changes, and they are stringently linked, all-or-none markers of transformation.

Neoplasia is manifested by transformation that is heritable and characterizes every tumor cell. Since there is a shared program of commitment to proliferation in all cancer cells, displayed as the malignant transformation, the activities of certain enzymes and the concentrations of strategic metabolites should exhibit the same type of alterations in all tumors in a spectrum of neoplasms. Such all-or-none alterations, stringently linked with...
transformation, do occur in tumors. Thus, the activities of certain key enzymes are increased, and the activities of other enzymes functionally opposed to them are decreased in all the neoplasms.

**Coincidental Alterations: The Apparent Diversity.** In Class 3 are alterations that fluctuated without relation to malignancy. These coincidental changes in gene expression are not stringently linked with neoplasia.

Alterations in certain enzymic activities that showed no relation to transformation or progression in the tumor spectrum proved to be due to the behavior of non-key enzymes that are present in excess. Regulation of their activities is not linked with neoplasia.

**Ordered Pattern versus Diversity and Randomness.** The behavior of the activities of key enzymes revealed an ordered transformation- and progression-linked reprogramming of gene expression. The meaningful pattern of enzymic and metabolic imbalance was first identified in the spectrum of hepatomas of different proliferative rates. It was recognized that not all enzymes should be expected to be linked to transformation and progression because the integration of gene expression operates through the control of a relatively small number of key enzymes. Some alterations showed no pattern, since they involved enzymes or metabolites present in an excess. Such apparent diversity and randomness indicate coincidental alterations in gene expression that are not stringently linked to the core of neoplasia. What is important about cancer is ordered; what is not is the random element and the diversity (97, 98).

**Operational Advantages of the Molecular Correlation Concept: A Unifying Approach in Cancer Biochemistry.** The operational advantage of the molecular correlation concept is its testability; it provided predictions that could be verified or disproved. That a theory must be testable to be useful, and thus not is the random element and the diversity (97, 98).

Observations in carbohydrate metabolism and conceptual advances led to the recognition that not all enzymes should be expected to be stringently linked with transformation and progression because the integration of gene expression operates through the stringent control of a relatively small number of enzymes (97, 98). I termed these key enzymes because the regulation of the rate and direction of the flux of opposing and competing synthetic and catabolic pathways is achieved through their control (97, 98).

**The Key Enzyme Concept**

The concept of key enzymes was developed in this laboratory along with methods for identifying such enzymes (97, 98). Earlier studies on tumor metabolism which failed to detect a meaningful pattern were due, in part at least, to the poor choice of enzymes and other biochemical variables. Our introduction of the concept of key enzymes provided an approach to clarify the strategy of gene expression in normal and neoplastic cells (92, 93, 97, 98, 114, 121).

The key enzymes usually have relatively low activities, govern one-way metabolic reactions, and frequently are the first or the last in reaction sequences; they may be pathways in themselves or operate on both sides of reversible reactions with a one-way enzyme opposed by another one-way enzyme. Key enzymes are frequently the targets of feedback or multiple regulation, exhibit allosteric properties, or are interconvertible enzymes and may have an isozyme pattern (97, 98). The activities and amount of key enzymes are frequently subject to nutritional and hormonal regulation. The behavior of activities, amount, and isozyme pattern of key enzymes is stringently linked with transformation and progression in cancer cells. Examples include: in pyrimidine metabolism, r,3'-amidopyl-phosphate synthetase II, CTP synthetase, ribonucleotide reductase, DNA polymerase, dihydrothymine dehydrogenase; in purine metabolism, amidophosphoribosyltransferase, xanthine oxidase, IMP dehydrogenase; in carbohydrate metabolism, glucokinase-hexokinase, 6-phosphofructokinase, pyruvate kinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase.

**The Non-Key Enzymes.** These are enzymes that apparently do not become limiting in metabolic flux. Such enzymes are usually characterized by the following properties: they catalyze reversible equilibrium reactions and are shared by both synthetic and catabolic pathways; they exhibit high activities; and they are present in excess. Their activities are usually not markedly altered by nutritional and endocrine regulation and do not correlate with transformation or progression in neoplasia. Examples include: in pyrimidine metabolism, dihydroorotate oxidase; in
purine metabolism, GMP kinase; and in carbohydrate metabolism, phosphohexoseisomerase, lactate dehydrogenase, 6-phosphogluconate dehydrogenase.

**Testable Predictions.** It was an important advance in our conceptual approach when we recognized the key enzymes because testable predictions could be made regarding their expected behavior in neoplastic transformation and progression. Analysis of behavior and properties of the key enzymes and their relation to transformation and to progression in a spectrum of neoplasms has permitted identification of the linking of biochemical alterations with the biological behavior of tumors.

**Information Value of Enzyme Activity Determinations.** The following information may be expected from assaying key enzymic activities in normal and cancer cells.

1. Under optimum assay conditions, when the only limiting factor is the amount of enzyme, it is assumed that the elevated or lowered specific activity is an accurate reflection of the enzyme amount. The validity of this assumption was confirmed by immunotitration for 5 enzymes from different metabolic pathways (19, 50, 79, 87, 88) (see below).

2. Measurement of enzymic activity may be utilized in probing the pattern of gene expression in normal and cancer cells (97, 98). Determination of enzyme concentration demonstrates quantitative alterations in gene expression. In the hepatoma model system, it has revealed a reprogramming of gene expression in different metabolic areas.

3. By measuring the isozyme pattern, qualitative aspects of gene expression can be elucidated. Our studies indicated that there was a definite pattern, which I named the isozyme shift (97, 98). The shift was evidenced by the loss of the liver-type, high-Km enzymes that were strongly regulated by nutritional and hormonal controls. These were largely or completely replaced by muscle-type isozymes that had low Km and high affinity for substrate and were less or not at all subject to physiological (nutritional and endocrine) regulation and feedback signals and required no activator. Examples of these in the hepatomas are the replacement of glucokinase with hexokinase (127, 128), liver-type pyruvate kinase with muscle-type isozyme, liver-type AMP deaminase chiefly by the muscle-type enzyme (36), and liver-type, high-Km, uridine kinase with low-Km, uridine kinase isozymes. Important work was carried out in the isozyme area by Weihouse and his associates that illuminated the isozyme pattern of hepatomas and other critical areas of cancer biochemistry (126–128).

4. Key enzymes are indicators of the activity of the overall metabolic pathway. To test the validity of this assumption, we compared the activities of key enzymes and their behavior with that of the relevant overall metabolic pathways and with concentrations of end products, intermediates, key metabolites, and nucleotides. There was good agreement between the alterations in the behavior of the key enzyme activity and in that of the activity of the metabolic pathway (90, 92). In the hepatoma spectrum, the increased activities of key enzymes of glycolysis measured in extracts were paralleled by an increase in glycolysis; conversely, the decreased activities of the key gluconeogenic enzymes was reflected in the decrease in the gluconeogenic pathway. The activities of these pathways were measured in liver and hepatoma tissue slices by determining the conversion of glucose to pyruvate and lactate and by the production of glucose from pyruvate (84). The elevated activity of a key enzyme of pentose phosphate production (glucose-6-phosphate dehydrogenase) is in line with the capacity for the direct oxidative pathway which increased in hepatomas, as determined by isotope studies in slices (90, 92). The stringently linked correlation of thymidine kinase activity (measured in extracts) with hepatoma growth rate was paralleled with the transformation and progression linked rise in the behavior of the overall synthetic pathway measured in tissue slices by the incorporation of thymidine to DNA. Conversely, the decline of the activity of the rate-limiting enzyme of thymidine degradation, dihydrothymine dehydrogenase (assayed in extracts), was paralleled by the decrease in activity of the degradation of thymidine to CO2, measured in liver and hepatoma tissue slices (23, 97, 98, 123). The stepped-up activity of CTP synthetase (assayed in extracts) is reflected in the increased concentration of CTP (measured in freeze-clamp tissue samples), and both parameters were transformation and progression linked (34, 47). The increase in GMP concentration reflects the elevated activity of GMP synthetase (10, 34). The progression-linked depletion in the concentration of L-glutamine in the hepatoma spectrum is paralleled with the rise in the activity of enzymes involved in glutamine utilization in the tumors (Table 8). Similar correlations have been noted in relating the decrease in activities of urea cycle enzymes with the decrease in urea cycle activity and the increase of activities of key enzymes of polyamine biosynthesis with the elevation of metabolites of polyamine production (122, 131).

Such correlations can be expected only if the activities of key enzymes, particularly the rate-limiting ones, are compared with the activity of the relevant metabolic pathway. For instance, the activity of the rate-limiting enzyme, hexokinase, correlated with the behavior of glycolysis, however, the activities of phosphohexoseisomerase or lactate dehydrogenase, which are present manyfold in excess in the liver, provided no correlation or pattern.

5. Since key enzymes are targets of chemotherapy, determination of the activity of such enzymes after drug treatment may provide useful information on the extent of inhibition achieved by the anticancer therapy (17, 120). A lack of response or the presence of enzymatic activities markedly higher than those usually observed in that type of tumor may indicate the emergence of resistance due to enzyme overproduction.

6. Since enzymes, particularly salvage ones, play a role in activation of certain antimetabolites, in case of development of drug resistance decrease or deletion in activities of such activating enzymes might reveal the mechanism of drug resistance which includes gene amplification. On the other hand, the presence of such activating enzymes indicates the feasibility of treatment with a drug that required activation by the enzyme.

7. Since 43 enzymic activities were recognized as transformation linked, these enzymes may be used as markers of neoplastic transformation and should provide assistance in the biochemical diagnosis of neoplasia.

8. Since 28 enzymic activities were recognized as progression linked, these enzymes may be utilized as markers of tumor malignancy, and they may be of assistance in the biochemical grading of malignancy.

**Biological Model Systems**

In 1955, we showed that during feeding of a hepatocarcinogenic dye to rats the activity of liver glucose-6-phosphatase steadily decreased and was absent in the resulting primary hepatocellular carcinomas (103). Other investigators, including
Goldfarb and Pugh (24), in an excellent recent study, confirmed these observations. In 1957, we were the first to show the absence of an enzymic activity in transplantable cancer cells when we demonstrated that glucose-6-phosphatase activity was absent in the chemically induced, transplantable, rapidly growing Novikoff hepatoma (105). We also reported that in the Novikoff hepatoma glucose-6-phosphatase activity could not be induced by starvation or steroid treatment which increased the enzymic activity in normal and host liver (105). These studies were the first to indicate the lack of responsiveness of a tumor enzyme-forming system to nutritional or hormonal regulation.

In 1959, it was reported that the Dunning hepatoma had some glucose-6-phosphatase activity (67). Since the Dunning had a slower growth rate and was better differentiated than the Novikoff hepatoma, it seemed to me that the different growth rates and malignancy of tumors should be manifested also in gradations in the expression of biochemical properties (106). To test this idea rigorously, there was a need for hepatocellular carcinomas of different growth rates, degrees of differentiation, and malignancy. The first of such to come to my attention was the chemically induced transplantable hepatoma 5123D which (in 1960) was a slowly growing, well-differentiated tumor. The biochemistry of this tumor when compared with that of the Novikoff hepatoma confirmed the expectation I expressed in 1959 that a spectrum of tumors of different malignancy would be helpful in understanding the biochemistry of cancer cells (106). Subsequently, other hepatomas of different malignancy became available, and from these I assembled a spectrum of hepatocellular carcinomas in which the molecular correlation concept was tested (115).

Most of these tumors I obtained originally from Dr. Harold P. Morris, who was then at the National Cancer Institute and later at Howard University. It is a pleasure and a privilege to acknowledge the harmonious, productive collaboration with Dr. Morris that spanned over 20 years, involved many thousands of these tumors, and resulted in 50 joint publications. The Morris hepatomas were made available to me and to about 60 laboratories in the United States and around the world, and they have been of immense use in many aspects of cancer research. Through producing, maintaining, and distributing these tumors, many of them in collaborative research, Dr. Morris has made an imperishable contribution to cancer research.

**The Measure of Malignancy in the Tumor Spectrum.** In the hepatoma spectrum, growth rate is used as a measure of the degree of malignancy. The rate of proliferation was quantitated by several independent techniques: biological (tumor size and volume); cytological (mitotic count); histological (degree of differentiation); and biochemical (thymidine index). The malignancy of the tumor lines was ranked also by time required in weeks for the neoplasms to reach a uniform diameter of 1.5 cm (97, 98). All methods yielded the same ranking orders for the tumors (61, 62). Most of these solid tumor lines showed remarkable stability which indicated a stable gene pool and permitted good reproducibility of the studies in many laboratories (97, 98). The mathematical relation between tumor-proliferative rate and the extent of alterations in biochemistry is measured by correlation coefficients.

**Operational Advantages of the Hepatoma Spectrum as a Model System for Testing the Molecular Correlation Concept.** The spectrum consisted of lines of transplantable solid hepatomas, each with a different proliferative rate. 1. Since all were of the same cell type, hepatocellular carcinomas, they provided the 2 biological properties we wished to characterize. (a) All tumors were composed of transformed cells and therefore testable for the behavior of the activities of enzymic and metabolic parameters that were altered in all tumors. This permitted the discovery of transformation-linked alterations. (b) Since the tumor spectrum consisted of neoplasms with increasing degrees in the expression of malignancy (increased proliferative rate and decreased differentiation), the biochemical properties that were linked with neoplastic progression could be identified.

2. Another advantage was that most of these tumors consisted of 80 to 95% neoplastic cells with relatively little stroma, a cellular population much more homogeneous than that of primary tumors.

3. Because the tumors were of different growth rates, the spectrum permitted elucidation of the biochemical basis of proliferation rate.

4. Since responsiveness of human neoplasms to chemotherapy is determined in part at least by their growth rate (136), these slowly and rapidly growing rat hepatomas offered an opportunity to clarify the biochemical basis of drug responsiveness.

5. The slowly growing hepatomas are useful models for the human disease which is a major clinical challenge to chemotherapy. On a global scale, liver tumors are the most common neoplasms (9).

6. The biological and biochemical properties of many of the different tumor lines were remarkably unchanged from generation to generation. This stability was due, in large part, to the fact that the tumors were originally transplanted and carried in the laboratory of Dr. H. P. Morris. The stability of the variation in the different tumor lines can be determined by karyotypic, enzymic, or histological assays, and these can be related to the proliferative rate of the tumors.

7. An important operational advantage of the liver tumor spectrum is the availability of appropriate, meaningful control tissues (normal, regenerating, and differentiating liver). Dedifferentiated tumors for which no control tissue is available (Ehrlich tumor, HeLa cells, etc.) are of limited interpretive value in this context.

8. An advantage of much interest to biochemists and pharmacologists is that from many of these hepatomas an almost unlimited quantity of tumor material is available, which is helpful in enzyme purification and in pharmacology. Because of its uniform growth rate and retention of a high percentage of viable cells, hepatoma 3924A is particularly suitable for freeze-clamp studies.

9. It might be argued that the progression-linked alterations occurred only in the proliferating cells that are part of the heterogeneous cell populations of these tumors. This would suggest that the enzymic and metabolic alterations do not occur in all cells, but only in those in the growth fraction; i.e., the enzymic increases would indicate merely the presence of an expanding growth fraction. In some of the hepatomas, the growth fraction was measured (54, 78). If such reasoning were valid, then the biochemical assays would provide only an indirect way of measuring the growth fraction.

This argument is refuted by extensive documentation that a number of enzymic and metabolic changes occur to about the same extent in every tumor examined in the spectrum, including...
the slowly growing (with the lowest growth fraction) and the most rapidly growing (highest growth fraction). Such transformation-linked alterations indicate that the biochemical changes are present in all or nearly all cancer cells in the various tumors.  

10. Since the most rapidly growing hepatomas required 1 week, whereas the slowest growing ones took 52 weeks, to reach a diameter of 1.5 cm, the hepatoma spectrum provided an array of neoplasms of vastly different proliferative rates and degrees of differentiation. In the very slowly growing hepatomas, the threshold alterations, and in the most rapidly growing tumors, the extreme expression of biochemical imbalance can be identified. Such an unparalleled model system has been most useful in cancer research, and its maintenance and availability to all investigators remain of great scientific importance.

**Determination of Specificity to Neoplasia, Role of Control Tissues, and the Power of Interpretation**

Determination of specificity of the biochemical pattern to the neoplastic liver was made feasible by the ready availability of appropriate controls: normal, resting liver; regenerating liver; and differentiating, developing liver. With these systems, it was possible to elucidate quantitative and qualitative differences between the metabolism of hepatomas and that of control tissues. The spectrum of hepatomas of different growth rates and the array of control tissues vastly increased the power of interpreting whether the biochemical changes were due to neoplastic transformation and progression or to rapid growth rate or different degrees of differentiation which could be readily determined from the control tissues. Detailed characterizations have been published (92, 93, 96–98, 104, 105, 121, 124), and a concise description of the control system is provided below because it bears on the interpretation proposed.

**Control, Normal Tissues.** The enzymic and metabolic phenotype of the hepatomas was compared primarily with that of the normal liver of rats of the same sex, strain, age, weight, and nutritional status as the tumor-bearing ones. Animals were kept in individual cages with Purina fox chow and water available ad libitum and were killed between 9 and 11 a.m. Rats were under regulated cycles of light and darkness. Extensive background studies were carried out in normal animals to establish to what extent nutritional or hormonal variation may impact on the liver enzymic and metabolic activity. The liver of the tumor-bearing rat is not a suitable control tissue, because it was shown that it was subject to numerous unpredictable artifacts. Host liver should be used as a control for hepatomas of different growth rates only in clearly specified exceptions.

**Regenerating Liver.** Since the various liver tumor lines had different rates of proliferation, it was essential to clarify whether the biochemical pattern was due to neoplastic alteration and progression or whether it reflected rates of proliferation that occur in normal liver. The biochemical phenotype of the proliferating normal liver was elucidated by studying the regenerating liver at different time periods after partial hepatectomy in adult rats. The liver of sham-operated normal rats was used as a control. The proliferation rate of the regenerating liver at 24 hr after operation is similar to that of hepatoma 3924A.

**Differentiating Liver.** Since the hepatoma spectrum consisted of tumor lines of different proliferative rates and degrees of differentiation, it was necessary to determine the behavior of biochemical parameters in the differentiating liver of developing rats. This model also provides a control for rapid proliferative rate in the postnatal rat. We noted that livers should not be used before 6 days after birth because in the rat the fetal liver is largely a hemopoietic organ; the overwhelming proportion of hemopoietic cells to liver cells in fetal rat liver was documented in histological sections (104). Many of the enzymic changes from fetal to postnatal livers represent not altered enzymic activities but shifts in the cellular population (97, 98, 104).

**Generalization**

To determine whether the biochemical pattern of enzymic and metabolic imbalance originally discovered in the chemically induced, transplantable hepatomas of the rat does or does not apply to other neoplasms in animals and in humans, a series of experimental neoplasms and primary human tumors was examined.

**The Kidney Spectrum.** Because kidney is the only organ other than liver that has strong gluconeogenesis and high glucose-6-phosphatase activity, I asked Dr. Morris to produce renal cell carcinomas for my laboratory. In these tumors, we tested the hypothesis whether the decrease in gluconeogenesis and in activities of gluconeogenic enzymes also occurs in renal neoplasia where glycolysis and gluconeogenesis operate as opposing pathways. The biological properties and histology of these tumors were reported (63); and in a series of papers, we elucidated the enzymology, nucleotide content, and metabolism of kidney tumors (33, 99, 107, 110, 132).

**Colon Carcinomas and Muscle Sarcoma.** The applicability of the biochemical imbalance discovered in hepatomas was studied in various other animal model systems; these include a chemically induced muscle sarcoma and a series of chemically induced colon tumors in mouse and rat (102, 111).

**Human Neoplasms.** To test the applicability to human malignancy of the biochemical alterations observed in chemically induced tumors in animals, primary neoplasms in human were investigated with emphasis on primary hepatocellular carcinoma (114), renal cell carcinoma (33, 69, 99, 107), colon carcinoma (30, 113), lung neoplasia (25, 118), and leukemic cells (7). Xenografts of colon carcinomas of different proliferative rates in the nude mouse were also studied (108). The special problems in obtaining comparable human normal tissues and the precautions that we take to achieve valid comparisons were outlined (33, 99).

**Pyrimidine Metabolism: Transformation- and Progression-linked Imbalance in Cancer Cells**

The biochemical strategy of cancer cells in pyrimidine and DNA metabolism is expressed in the behavior of the activities of opposing key enzymes and pathways of synthesis and catabolism and of the concentrations of crucial nucleotides and deoxynucleotides.

**Characteristic Features of Normal Liver Pyrimidine Metabolism.** The special features of pyrimidine metabolism in normal rat liver include the following.

1. The **de novo** synthetic pathway assembles UMP from precursors through 9 reactions. Carbamoyl-phosphate synthetase II is the first rate-limiting enzyme of UMP biosynthesis. The first 3 and the final 2 enzymes are each on a complex protein (43, 45). With the exception of dihydroorotate dehydrogenase which
Chart 1. Intracellular distribution of enzymes of de novo CTP synthesis in liver cells and the targets of antimetabolite action. A cytoplasmic protein which has 3 enzymic activities initiates the pathway, and a protein with 2 enzymic activities completes the series of steps leading to UMP biosynthesis. In addition to these 2 cytoplasmic complex proteins, comprising 5 enzymic activities, there is also dihydroorotate oxidase which is located on the outside of the inner mitochondrial membrane. From UMP, 2 reactions are catalyzed by kinases, yielding UDP and UTP. The subsequent enzyme, CTP synthetase, forms CTP; they are all cytoplasmic enzymes. OMP DC, orotidine-5'-monophosphate decarboxylase; OMP, orotidine 5'-monophosphate; OPRT, orotate phosphoribosyltransferase; OA, oxidase; DHO DH, dihydroorotate dehydrogenase; DHO, dihydroorotate; DHOase, dihydroorotase; CA~asp, carbamoylaspartate; ATCase, aspartate carbamoyltransferase; PALA, N-(phosphonacetyl)-L-aspartate; CA~P, carbamoylphosphate; CP-synth. II, carbamoyl-phosphate synthetase II.

is in the mitochondria, the other enzyme activities are in the cytosol (Chart 1). From UMP through actions of UDP and UMP kinases and CTP synthetase, the nucleotides UDP, UTP, and CTP are synthesized. The rate-limiting enzyme of the overall process of de novo CTP biosynthesis is CTP synthetase (Chart 2).

2. From the pyrimidine nucleotides, UDP and CDP, ribonucleotide reductase yields UDP and CTP. From subsequent synthetic steps of the de novo pathway, dCTP and dTTP are produced which, in the presence of dGTP and dATP, derived from the purine-synthetic pathways, provide the substrates for DNA polymerase for the biosynthesis of DNA. The rate-limiting synthetic enzyme of the entire de novo pyrimidine, purine, and DNA synthesis is ribonucleotide reductase which has orders of magnitude lower activity than any other enzyme in these metabolic areas (85) (see below and Table 1).

3. The salvage enzymes provide powerful alternative salvage routes to produce the key metabolites, UDP, UMP, and dCTP (Charts 3 and 8; see below): (a) uridine is salvaged by uridine-cytidine kinase; (b) uracil is recycled by uridine phosphorylase which produces uridine; (c) uracil is also recycled in a one-step process by the uracil phosphoribosyltransferase reaction; (d) uridine-cytidine kinase salvages cytidine to yield CMP, and CMP kinase produces CDP, the immediate precursors for CTP and also for CDP reductase from which the dCDP and dTTP are produced which, in the presence of dGTP and dATP, derived from the purine-synthetic pathways, provide the substrates for DNA polymerase for the biosynthesis of DNA. The rate-limiting synthetic enzyme of the entire de novo pyrimidine, purine, and DNA synthesis is ribonucleotide reductase which has orders of magnitude lower activity than any other enzyme in these metabolic areas (85) (see below and Table 1).

The abbreviations used are: dNTP, deoxynucleotide triphosphate; PRPP, phosphoribosylpyrophosphate (5-phosphoribosyl 1-pyrophosphate); XMP, xanthosine monophosphate; APRT, adenine phosphoribosyltransferase; GR, guanine phosphoribosyltransferase; CPS II, carbamoyl-phosphate synthetase II.
thymidine by thymidine kinase; this provides an alternative pathway for the production of the key deoxynucleotide precursor, dTMP.

4. The pyrimidine-synthetic pathways are opposed by the 2 main catabolic pathways that degrade thymidine and uridine to CO₂ and ammonia. The rate-limiting catabolic enzyme is dihydrothymine dehydrogenase which also catalyzes the dihydouracil dehydrogenase step. Recently, this enzyme was purified in this laboratory (81). It is important for an understanding of the biochemical strategy of cancer cells that in normal liver the capacity of the pyrimidine-degradative pathway is markedly higher than that of the rate-limiting enzymes of the synthetic pathways (23).

The Neoplastic Program of Pyrimidine Metabolism. In the neoplastic liver cells, there is a program expressed in pyrimidine metabolism which entails an increase in the activities of key enzymes of de novo biosynthesis and of salvage pathways concurrent with a decrease in activities of key enzymes of pyrimidine degradation (23, 58, 82, 94, 95, 97, 98, 121, 123).

Here, characteristic examples will be given of the behavior of the enzymes that are transformation and progression linked.

The specific activity of carbamoyl-phosphate synthetase II in 17 rat hepatomas was significantly increased above the activity observed in control livers. The rise in enzyme activity correlated positively with the increase in the proliferative rates of the tumors, yielding a Spearman’s rank correlation coefficient (rₛ) of 0.924, significant at the 1% level (Chart 4). In the slowly growing hepatomas, the increases in activities were 1.3- to 2.9-fold; in the rapidly growing hepatomas, they were 5.7- to 9.5-fold higher than in normal control liver. The molecular correlation concept classified the behavior of this enzyme as transformation linked because the activity increased in all the tumors, even the slowest ones, and it was progression linked because it correlated significantly with the proliferation rate of the neoplasms (1, 3).

Carbamoyl-phosphate synthetase II exists as a multienzyme complex with aspartate carbamoyltransferase and dihydroorotase, the second and third enzymes of de novo uridylate synthesis (see Ref. 3). In analyzing 12 to 17 hepatoma lines, we found that there was a display of an integrated program in which the activities of other enzymes of de novo UTP synthesis (aspartate carbamoyltransferase, dihydroorotase, orotate phosphoribosyltransferase, and orotidine-5’-phosphate decarboxylase) increased (123). The activities of the first 3 enzymes were transformation and progression linked, and those of the final 2 enzymes were transformation linked (1, 3, 123). The activity of dihydroorotate dehydrogenase did not yield a pattern.

In 13 hepatomas of vastly different proliferative rates, we showed that the specific activities of UDP kinase (nucleoside-diphosphate kinase; ATP:UDP phosphotransferase) increased significantly (1.6- to 3.9-fold) in all neoplasms (Chart 5). Since the activity was elevated even in the slowest growing, most differentiated hepatomas, but without a relation with proliferative rate, this enzymic behavior was classified as transformation linked (130).

UDP kinase provides the substrate, UTP, for the rate-limiting enzyme of CTP biosynthesis, CTP synthetase, the specific activity of which is transformation and progression linked in the hepatoma spectrum (47). CTP synthetase activities in 14 tumors with slow and medium growth rates increased 1.6- to 4.4-fold and in rapidly growing hepatomas 5.4- to 10.6-fold over the activities of the normal liver, yielding a significant Spearman’s rank correlation coefficient (47).

The most profound increase in an enzymic activity measured
thus far was that of ribonucleotide reductase in the hepatomas. Elford et al. (21) reported a marked rise of the reductase activity in hepatomas; the increase paralleled the tumor growth rates. However, the relationship with the activity of the normal liver could be determined only when Takeda and Weber (85) improved the enzyme assay to achieve a sensitivity which was capable of detecting and measuring precisely CDP reductase activity in normal, resting adult rat liver (23 pmol/hr/mg protein). The stringent linkage of CDP reductase activity with neoplastic proliferation is shown in Chart 6. The reductase activity increased 7.7- to 15-fold in the slowly growing hepatomas and 123- to 325-fold in the rapidly growing tumors. Thus, the behavior of CDP reductase activity was both transformation and progression linked.

DNA polymerase, which has the second lowest activity in pyrimidine synthesis, occupies a strategic role in the immediate utilization of dNTPs for DNA biosynthesis. Laszlo and his associates showed that the activity of this enzyme was markedly increased in the hepatoma spectrum and that the rise correlated positively with the increase in hepatoma growth rate (6, 66). An increase in correlation with hepatoma growth rate was also observed in the activity of RNA polymerase which utilizes the ribonucleoside triphosphates for RNA biosynthesis (18).

The Salvage Enzymes: Transformation- and Progression-linked Increase. An important component in the biochemical strategy of cancer cells poses a major problem in the design of chemotherapy, namely, that the activities of all the salvage enzymes increased in pyrimidine metabolism (Chart 3). It is particularly relevant that in pyrimidine production the activities of the salvage enzymes were higher than those of the rate-limiting enzymes. The specific activities of the rate-limiting enzymes of de novo UMP, CTP, and dTMP synthesis range from 23 pmol to 9 mol/hr/mg protein in the liver, whereas the salvage enzymes could synthesize 0.8 to 156 nmol products. In the rapidly growing hepatoma, the increased activities of the rate-limiting enzymes of de novo synthesis could produce 4.2 to 86 whereas salvage enzymes could synthesize 11 to over 1000 nmol/hr/mg protein. This is especially striking for the activity of uridine-cytidine kinase that is increased 2- to 3-fold in all the examined slow and intermediate growth rate hepatomas and 5- to 6-fold in the rapidly growing ones. These are transformation-linked increases. The specific activities of uridine phosphorylase and uracil phosphoryl-bosyltransferase increased in a similar fashion (123). The activity of deoxycytidine kinase increased in all the hepatomas, and in the rapidly growing hepatomas 12- to 18-fold over that in normal control liver (27) (Chart 7). This is important because deoxycytidine kinase channels the nucleoside deoxycytidine directly into the deoxynucleoside monophosphate pools, circumventing the ribonucleotide reductase reaction (Chart 8).

Thymidine metabolism is most relevant to DNA synthesis (51, 97, 98). Thymidine is salvaged by thymidine kinase, providing an alternate route for dTMP production for DNA biosynthesis. Thymidine may be degraded by thymidine phosphorylase and through the activity of the rate-limiting enzyme of thymidine
catabolism, dihydrothymine dehydrogenase, for eventual catabolism to CO₂ and ammonia. Since thymidine phosphorylase is an equilibrium enzyme, the fate of thymidine depends on the balance of the activities of thymidine kinase and dihydrothymine dehydrogenase. In all hepatomas, thymidine kinase activity increased in parallel with the proliferative rate of the tumors (123). In the rapidly growing hepatomas, the activity of thymidine kinase was elevated to over 300-fold that of the normal liver. In contrast, the decreased activity of dihydrothymine dehydrogenase in all hepatomas correlated negatively with the proliferative rate of the tumors (121, 123). Thus, the behavior of the kinase and the dehydrogenase was transformation and progression linked (Chart 9).

The activities of the overall metabolic pathways, both synthetic (incorporation of thymidine into DNA) and catabolic (degradation of thymidine to CO₂), were measured in liver and hepatoma slices. The rise in the activity of the synthetic pathway closely paralleled the increase in thymidine kinase activity, and the decline in the activity of the catabolic pathway closely paralleled the decrease in dihydrothymine dehydrogenase activity (23, 123).

The opposing behavior of the activities of the key antagonistic enzymes and overall pathways of pyrimidine synthesis and catabolism is an example of reciprocal regulation of metabolic pathways and key enzymes. It is in line with the opposing behavior of antagonistic enzymes and pathways in other metabolic areas, including glycolysis and gluconeogenesis, purine synthesis and degradation (97, 98, 123, 125). Since the activity of the catabolic pathway was orders of magnitude higher than that of the synthetic one, the decrease in thymidine catabolism was as important as the rise in utilization of thymidine for DNA synthesis (23, 123). The decrease in the activity of the degradative pathway in hepatomas was accounted for, in part at least, by the decrease in the activity of the rate-limiting enzyme, dihydrothymine dehydrogenase (121), and by the decline in the concentration of NADPH (77), the cofactor for the dehydrogenase reaction.

These observations reveal the ordered pattern of pyrimidine metabolic imbalance in cancer cells. The reciprocal changes in the activities of opposing key enzymes and overall synthetic and catabolic pathways should amplify the metabolic imbalance. The altered biochemical phenotype indicates the operation of a genetic program that is part of the pleiotropic strategy of cancer cells.

Biochemical Commitment to Neoplastic Replication in Pyrimidine Metabolism. The biochemical strategy of cancer cells
is expressed in pyrimidine metabolism in the integrated imbalance of the activities of key enzymes and metabolic pathways of de novo and salvage biosynthesis and in the decline in the activities of the key enzymes and the overall activity of pyrimidine degradation (thymidine to CO₂). The integrated imbalance is summarized in Chart 10. It is important to note that the targets of experimental and clinical anticancer drugs are usually the key enzymes of the de novo synthetic pathways. It is a purpose of this presentation to bring into focus the need to block also salvage biosynthesis by inhibiting the activity of specific salvage enzymes or the transport of salvage nucleoside precursors.

Table 1 shows the specific activities in normal liver of the synthetic and catabolic enzymes of pyrimidine and DNA metabolism. The enzymes with the lowest activities in liver of normal adult rat were elevated to the highest extent in the rapidly growing hepatoma. In contrast, the synthetic enzymes with the highest activity in normal liver had the smallest extent of rise in the rapidly growing tumor. It is important that the rate-limiting enzyme of thymidine or uridine degradation, dihydrothymine dehydrogenase, had much higher activity than the rate-limiting synthetic enzyme of the de novo pathway, CDP reductase, or that of the enzyme with the second lowest activity, DNA polymerase. Thus, in resting liver, the metabolic balance should favor the catabolism of uridine and thymidine. The strategy of the cancer cell entails not only marked transformation- and progression-linked increases in the activities of the key synthetic enzymes but also transformation- and progression-linked decreases in the activity of the rate-limiting catabolic enzyme. These integrated multi-enzyme alterations reveal the enzymic strategies of the cancer cell as displayed in pyrimidine and DNA metabolism (Chart 10).

Purine Metabolism: Transformation- and Progression-linked Imbalance in Cancer Cells

Characteristic Features of Normal Liver Purine Metabolism. In liver purine metabolism, the following special features are relevant (see Charts 14 and 15 and Table 2).

1. The de novo synthetic pathway assembles from precursors through 10 reactions the key purine nucleotide, IMP, from which adenylsuccinate, AMP, ADP, ATP, XMP, GMP, GDP, and GTP are produced. AMP may be converted to IMP by AMP deaminase; a purine nucleotide cycle operates in the liver. The key purine nucleotide, IMP, may be produced through (a) de novo biosynthesis, (b) the purine nucleotide cycle, and (c) the salvage reaction catalyzed by hypoxanthine-guanine phosphoribosyltransferase activity (see below).

2. From GDP and ADP, through ribonucleotide reductase, dGDP and dADP are produced from which the kinase synthesizes dGTP and dATP. From these purine dNTPs, in the presence of dCTP and dTTP produced by the pyrimidine-synthetic pathways, DNA polymerase synthesizes DNA. GTP and ATP, in the presence of CTP and UTP, are converted by RNA polymerase into RNA. The rate-limiting enzyme for the de novo biosynthesis of IMP is the first one, amidophosphoribosyltransferase; in the utilization of IMP for adenylate biosynthesis, it is adenylosuccinate synthetase, Whereas for the utilization of IMP for guanylate biosynthesis, it is adenylosuccinate synthetase, whereas for the utilization of IMP for guanylate production it is IMP dehydrogenase which also has the lowest
activity of all the purine enzymes.

3. The highly active salvage enzymes provide alternative routes to the *de novo* synthesis to produce the strategic purine nucleotides, IMP, AMP, and GMP. Two salvage enzymes act to recycle 3 purine nucleoside precursors (hypoxanthine, adenine, and guanine) to nucleotides in the presence of PRPP. APRT converts adenine to AMP. Because the activities and the *K*ₐ₅ of the other salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase, differed with hypoxanthine or guanine as substrate, for convenience of discussion the activities are reported separately as HPRT and GPRT (112).

4. The *de novo* and salvage pathways of IMP synthesis are opposed by the catabolic pathway. IMP is degraded to uric acid and, in the rat, to allantoin through the subsequent actions of 5'-nucleotidase, inosine phosphorylase, xanthine oxidase (Chart 11), and uricase (Table 2). In rat liver, the activity of the rate-limiting catabolic enzyme, xanthine oxidase, is higher than that of the rate-limiting enzyme of *de novo* biosynthesis, amidophosphoribosyltransferase (71, 72).

The Neoplastic Program in Purine Metabolism

**De Novo IMP Biosynthesis and Degradation.** The neoplastic program entails a transformation-linked increase in the activity of amidophosphoribosyltransferase (71) (Chart 11) and a transformation- and progression-linked rise in that of formylglycinamidine ribonucleotide synthetase.³ By contrast, there is a transformation-linked decrease in the activities of the enzymes of IMP catabolism. Thus, in all hepatomas, the activities of 5'-nucleotidase, inosine phosphorylase, xanthine oxidase (Chart 11), and uricase (46, 72, 119) (Chart 16; Table 2) were decreased.

**De Novo Guanulate Biosynthesis from IMP.** In the utilization of IMP for *de novo* guanulate biosynthesis, the activities of IMP dehydrogenase (Chart 12) and GMP synthetase increased in all

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for which GDP-mannose is required (133) and also might limit the synthesis of cyclic guanosine 3'-5'-monophosphate. GTP is an important factor in protein synthesis, and its role in DNA biosynthesis has recently been pointed out (13). GTP is an activator of CTP synthetase (47) and adenylsuccinate synthetase (36). Thus, a drop in its concentration might curtail the biosynthesis of CTP and ATP. Conversely, because GTP is an inhibitor of AMP deaminase (36), a decrease in GTP pools would deplete AMP deaminase activity with a subsequent drain on AMP pools and a rise in IMP concentration. High levels of IMP could impair the fidelity of DNA transcription by blocking certain steps in the proofreading mechanisms. GTP is an activator of ribonucleotide reductase (85) and promotes the conversion of GDP into dGTP; a marked decline in the GTP and GDP pools could limit the synthesis of dGTP which in turn would result in an imbalance in the concentration of the 4 dNTPs, hence limiting DNA biosynthesis.

**De Novo Adenylate Biosynthesis from IMP.** In the utilization of IMP for de novo adenylate biosynthesis (Chart 16), the activities of adenylsuccinate synthetase and adenylsuccinate (adenylsuccinate lyase) increased in all the hepatomas examined, showing transformation-linked behavior (Chart 13) (36). The AMP produced may be utilized by the kinases for the biosynthesis of ADP and ATP and by adenylate kinase (15) to achieve a rapid equilibrium of adenylates. AMP may also be recycled to yield IMP by AMP deaminase activity which was markedly increased in the hepatomas and was transformation and progression linked (36) (Table 2).

**Salvage Activities for Production of AMP, IMP, and GMP.** In normal liver, the specific activities of the salvage enzymes APRT, HPRT, and GPRT (498, 460, and 1470 nmol/hr/mg protein, respectively) were 6.6- to 21-fold higher than that of amidophosphoribosyltransferase (70 nmol/hr/mg protein) (Chart 14) (74, 112). Thus, in resting liver, the capacity of salvage enzymes for recycling the bases was much higher than that of the rate-limiting de novo synthetic enzyme. A further metabolic

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**Figure captions:**

**Chart 12.** Transformation- and progression-linked increase in the behavior of the activity of IMP dehydrogenase in hepatomas of different growth rates. Mean specific activities are plotted as percentages of the normal liver values. Bars, S.E. All hepatoma activities were significantly higher than those of the normal liver (p < 0.05).

**Chart 13.** The transformation-linked increase in the activity of adenylosuccinase in hepatomas. Mean specific activities are plotted as percentages of the normal liver activity. Bars, S.E. All enzyme activity increases were significantly different from the values of the normal liver (p < 0.05).
advantage for the salvage pathways is that the affinity of the 3 salvage enzymes to PRPP is much better than that of amidophosphoribosyltransferase (112).

In rapidly growing hepatoma 3924A, the specific activity of the amidotransferase was increased 2.8-fold, whereas those of APRT, HPRT, and GPRT were 170, 70, and 70% of normal liver values (112). With these changes, the activity of the amidotransferase was 193 nmol/hr/mg protein, whereas those of the salvage enzymes were 4.4-, 1.7-, and 5.4-fold, respectively, of the amidotransferase activity. For the common substrate, PRPP, for amidotransferase, the \( K_m \) was 400 \( \mu M \); whereas for APRT, HPRT, and GPRT, the \( K_m \)’s were 2, 4, and 40 \( \mu M \), respectively (112). Accordingly, in the hepatoma, the activities of the salvage enzymes were markedly higher and the affinity for PRPP was much better than of the rate-limiting enzyme of de novo synthesis, amidotransferase.

A further advantage conferred to cancer cells by the reprogramming of gene expression in purine metabolism is in the behavior of the activity of the enzymes involved in hypoxanthine metabolism. In normal liver, the activity of the salvage enzyme, HPRT, was 460 nmol/hr/mg protein, whereas that of the degradative enzyme, xanthine oxidase, was 100 nmol/hr/mg protein, yielding a ratio of 4.6 in favor of the synthetic capacity. In hepatoma 3924A, with HPRT activity being slightly lower than in liver, but with xanthine oxidase activity decreased to 10%, a ratio of 33 was observed. This ratio should strongly favor utilization of hypoxanthine for salvage also because the \( K_m \)’s for hypoxanthine of HPRT and xanthine oxidase were similar, at 3 to 7 \( \mu M \). Similar shifts occur in the ratios of GPRT and guanine deaminase activities in liver and hepatoma (112, 117).

The relationship of activities of key enzymes of de novo and salvage synthesis of the strategic purine nucleotides, AMP, IMP, and GMP, is shown in Charts 14 and 15. In hepatoma 3924A, under optimum kinetic conditions, the amidotransferase could synthesize 193 nmol IMP per hr per mg protein. By contrast, HPRT could provide 325 nmol IMP, and this enzyme has a 100-fold better affinity for PRPP than did amidotransferase. In the routing of IMP to GTP, the rate-limiting enzyme is IMP dehydrogenase which under optimum conditions could yield 23 nmol XMP per hr per mg protein, whereas GPRT could produce 1020 nmol GMP per hr per mg protein. In the routing of IMP into adenylate synthesis, the rate-limiting enzyme, adenylosuccinicate synthetase, could synthesize 88 nmol adenylosuccinate per hr per mg protein; however, the salvage enzyme, APRT, could yield 951 nmol AMP per hr per mg protein (112).

As we have pointed out, the enzyme activities measured under optimum kinetic conditions indicate only an approximation of what actual cellular activities, which depend on the substrate, cofactor, and various regulatory metabolite concentrations in the tissue at the prevailing pH (112), might be. Thus far, isotope data are in line with the enzymic capacities (129). Further work is necessary to measure the activity of the metabolic fluxes which should determine the in vivo contributions of the de novo and salvage pathways. In the design and evaluation of chemotherapy, the effectiveness of inhibitors of enzymes of the de novo pathway may depend, in part at least, on the activities of salvage enzymes which could furnish the essential metabolites through highly active one-step recycling processes.

It is noteworthy that in the hepatomas the activities of key enzymes of de novo biosynthesis increased markedly, whereas the changes in activities of the salvage enzymes were minor. This may relate to the fact that in normal rat liver the purine salvage enzymes have high activities and high affinity for their substrates.

**Biochemical Commitment to Neoplastic Replication in Purine Metabolism.** The biochemical strategy of cancer cells in purine metabolism is expressed in the integrated imbalance of the activities of the key enzymes and metabolic pathways of de novo and salvage biosynthesis, and in the decrease in the activities of the key enzymes and overall activities of purine catabolism (Chart 16). The attacking points of experimental and clinical anticancer drugs are usually the key enzymes of the de novo synthetic pathways. It is an important objective of this paper to point out the need also to block salvage biosynthesis by inhibiting the activity of specific salvage enzymes or the transport of salvage precursors.

**The Pattern of Ribonucleotides and Deoxynucleotides in Hepatomas**

The \( \textit{in vivo} \) concentrations of AMP, ADP, and ATP were first measured in tumors in the application of the freeze-clamp technique to kidney neoplasms in studies that I carried out in Oxford, United Kingdom, in collaboration with H. A. Krebs, Marion Stubbs, D. H. Williamson, and others (132). We observed a decrease in the ATP concentration of the transplantable rat renal cell carcinomas. Similar results were soon obtained in my laboratory in hepatomas of different growth rates (124). Subsequent
investigations using high-pressure liquid chromatography confirmed these results and extended the analysis to 16 ribonucleotides (34). In the hepatoma spectrum, the only elevation in the ribonucleotide pools was the transformation- and progression-linked rise in CTP concentration which was 4-fold increased in hepatoma 3924A. The concentration of GMP was elevated in a transformation-linked fashion, and in hepatoma 3924A it increased 3.2-fold over the value of normal control liver. The ribonucleotides that participate in numerous metabolic reactions, including the nucleoside diphosphates serving as substrates for the ribonucleotide reductase reaction, occur in mm concentrations. In contrast, the dNTPs, which are primarily involved in DNA production, are present in the liver in μM concentrations; therefore, in tumors a major enlargement of the dNTP pools was expected. We determined that the increases in the dNTP pools were transformation and progression linked (Chart 17). In rapidly growing hepatoma 3924A, the concentrations of dATP, dGTP, dCTP, and dTTP increased to 17-, 5-, 8-, and 12-fold of those of normal liver. Chart 17 also shows that the concentrations of CDP, UDP, GDP, and ADP, substrates of the conversion of ribonucleotides to deoxynucleotides, were the same as in the liver across the hepatoma spectrum. However, the pools of these ribonucleotides were greatly in excess of those of the dNTPs. The concentrations of the substrates of ribonucleotide reductase, ADP, GDP, UDP, and CDP, were 1,060, 112, 210, and 56 nmol/g in liver and 778, 137, 249, and 50 nmol/g in hepatoma 3924A (34). In the presence of this great excess of substrates, the conversion of the ribonucleotides to deoxyribonucleotides apparently takes place only in traces in the resting liver due to the very low activity of ribonucleotide reductase. The transfor-
BEHAVIOR OF RIBONUCLEOSIDE DIPHOSPHATE POOLS

Chart 17. Progression-linked increase in the pools of dNTPs in hepatomas of different growth rates. It is noteworthy that the concentrations of the nucleoside diphosphates did not change across the hepatoma spectrum. *, alterations significantly different (Sig. diff.) from those observed in normal (Norm.) liver (p < 0.05). Data plotted from Jackson et al. (34). Med., medium.

In evaluating the significance of the alterations in enzymic activities, 2 assumptions were made: Assumption 1, that the activity of the enzyme measured in the presence of optimum substrate and cofactor and pH yielding linear kinetics was proportionate with the amount of the enzyme; Assumption 2, that the amount of enzyme was an indicator of the gene expression under the specified steady state conditions. Two independent methods were used to measure enzyme amount. Enzyme concentration was determined by (a) assay of enzymic activity where strict proportionality between activity and added amount of enzyme was ensured (kinetic evidence) and (b) immunotitration of the enzyme protein amount by specific antienzyme serum (immunological evidence). Key enzymes were selected for each of the 4 vitally important metabolic pathways that are profoundly altered in activity in rapidly growing hepatoma 3924A. The key enzymes were highly purified from rat liver and hepatoma 3924A and were used to produce antisera in rabbits. The specificity of the antiserum for each enzyme was determined, and immunotitration was carried out. The concentrations of a key enzyme of glycolysis (6-phosphofructokinase), of pentose phosphate production (glucose-6-phosphate dehydrogenase), of de novo purine biosynthesis (amidophosphoribosyltransferase), and of de novo pyrimidine biosynthesis (CTP synthetase) were elevated. This was shown both by assay of enzyme activity and by immunotitration of the amount of enzyme protein (19, 79, 87, 88) (Chart 18).

In addition, an important enzyme of pyrimidine salvage, thymidine kinase, was examined. This enzymic activity in slow, intermediate, and rapidly growing tumors (hepatomas 16, 7787, and 3924A) increased 5-, 16-, and 30-fold, and a 4-, 15-, and 20-fold excess of antiserum was required to neutralize 50% of the thymidine kinase activities. We concluded that the elevated thymidine kinase activity in rat hepatomas of different growth rates - and progression-linked increase of the deoxyribonucleotide pools in hepatomas of different proliferative rates emerges in the presence of an unchanged amount of the ribonucleoside diphosphates. The stringent linkage of ribonucleotide reductase activity with the rise in hepatoma proliferation rate and with the increase in the levels of dNTPs suggests that the increase in reductase activity accounts for the channeling of the ribonucleotides to the deoxynucleotide pools (85). The elevated activity of reductase (85), along with the increased activities of PRPP synthetase, CTP synthetase, thymidine kinase, and IMP dehydrogenase, provide an enzymic explanation for the expansion of the dNTP pools (116, 117). In the presence of an excess concentration of the substrates, the ribonucleoside diphosphates, the rise in the activity of the enzyme, ribonucleotide reductase, is responsible for the increased product formation, the striking enlargement in the dNTP pools.

Reprogramming of Gene Expression in Neoplastic Cells: The Evidence

It was proposed that the enzymic and metabolic pattern of ordered transformation- and progression-linked alterations in tumor cells was a manifestation of the reprogramming of gene expression (97, 98). This interpretation of the altered phenotype of cancer cells is based on demonstration of changed concentrations in the end products of gene expression: the amount of specific catalytic proteins, the enzymes.

![Graph](chart18.png)

Chart 18. Evidence for reprogramming of gene expression in cancer cells. Increased concentrations of key enzyme amounts in carbohydrate, pentose phosphate, purine, and pyrimidine metabolism. G-6-P dehydrogenase; glucose-6-phosphate dehydrogenase.
rates reflected the concordant increase in the concentration of the thymidine kinase protein (50).

The molecular events that resulted in this heritable multienzyme change in the phenotype are of the greatest interest (125). The molecular basis of the reprogramming of gene expression that resulted in the integrated alterations in activities of over 40 enzymes is under investigation.

Specificity to Cancer Cells of Enzymic and Metabolic Pattern

The developing and differentiating liver and regenerating and normal liver in adult rats provided appropriate control models. Although they have similar growth rates, these control tissues can be distinguished readily both from neoplastic liver and from each other by their enzymic and metabolic pattern (96–98). Examples of the discriminating power of the biochemical pattern are shown in Tables 3 and 4. There are alterations that overlap with those in neoplastic tissues; however, the enzymic and metabolic imbalance is characteristic of neoplasia, since there is no overall pattern similar to that in neoplasia (34, 91, 92, 96–98, 104, 110, 121, 124).

Generalization: Presence of a Strongly Conserved Segment of Gene Expression in Animal and Human Neoplasms

Similar Pattern of Gene Expression in Different Types of Tumors. An overall pattern of enzymic imbalance was discovered in chemically induced, transplantable rodent tumors, including hepatomas, kidney tumors (33, 69, 97, 98), and sarcoma in rats (106); colon carcinomas in rats and mice (111); and myeloma, lymphosarcoma, and lymphocytic leukemia in mice (110). Current work brought evidence for a similar enzymic pattern in the Lewis lung tumor in mouse.4 It is striking that the enzymic imbalance was also observed in the MC-29 virus-induced, transplantable hepatoma in chickens (49, 70, 83).

We determined the presence of the pattern in human hepatocellular (114) and renal cell carcinomas (33, 73, 99, 107, 110), in colon tumor xenografts of different degrees of differentiation and growth rates (108), and in human primary lung and colon adenocarcinomas (16, 118). Recently, part of the pattern was also observed in human leukemias and lymphomas (7, 22). The applicability of all or part of the metabolic imbalance was demonstrated in 16 different animal and human neoplasias (Table 5).

Independence of Biochemical Program of Cancer Cells from the Carcinogenic Agent. The altered enzymic pattern in rat, mouse, chicken, and human neoplasia identifies a strongly conserved segment of the altered gene expression in neoplastic cells. Therefore, it appears that essential elements of the biochemical commitment to neoplastic transformation and progression have been identified. The biochemical program displayed is independent from the carcinogen, since the tumors analyzed included those produced by chemical and viral agents and those that arose spontaneously, e.g., primary human neoplasia. The various carcinogens apparently trigger through a final common pathway the display of the enzymic and metabolic program that we have identified in different species and in tumors of different cell origin and histology.

Tumor-specific Biochemical Markers. Just as there is a general biochemical imbalance shared by many different types of tumors, there are also tumor type-specific biochemical markers that aid in pinpointing alterations that are characteristic of individual classes of tumors. An example is galactokinase activity which increased in every human primary colon tumor, but decreased in other neoplasms (16). Uridine phosphorylase activity increased in all other tumors examined but was low in colon carcinomas (16). In the sarcoma, the pools of uridylates were

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**Table 3**

<table>
<thead>
<tr>
<th>Markers of gene expression</th>
<th>Normal liver</th>
<th>Hepatoma (rapidly growing 3683F)</th>
<th>Newborn (6 days old)</th>
<th>Regenerating (24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine to DNA</td>
<td>100&lt;sup&gt;a&lt;/sup&gt; 3,900</td>
<td>1,224&lt;sup&gt;b&lt;/sup&gt;</td>
<td>910&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Thymidine to CO&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>64&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Thymidine to DNA/thymidine to CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>100&lt;sup&gt;b&lt;/sup&gt; 11,500,000</td>
<td>7,188&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1,450&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>DNA/cell</td>
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<td>87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Carboxymo-phospho-synthe-tase II</td>
<td>100&lt;sup&gt;c&lt;/sup&gt; 946</td>
<td>216&lt;sup&gt;c&lt;/sup&gt;</td>
<td>146&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Aspartate carboxymo-transfer-a se</td>
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<td>223&lt;sup&gt;c&lt;/sup&gt;</td>
<td>137&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>165&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>CTP synthetase</td>
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<td>200&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>102&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>1,683&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Ribonucleotide reductase</td>
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<td>487&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5,586&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Thymidine phosphorylase</td>
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<td>55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>126&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Dihydrouracil dehydrogenase</td>
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<td>48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Dihydrotthymine dehydrogenase</td>
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<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Thymidine kinase/dihydrotthi-mine dehydrogenase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt; 17,200</td>
<td>2,702&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3,359&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Specific activities (nmol/hr/mg protein) were expressed as percentage of normal adult liver values.

<sup>b</sup> Qualitative discriminant.

<sup>c</sup> Quantitative discriminant.

---

**Table 4**

<table>
<thead>
<tr>
<th>Markers of gene expression</th>
<th>Normal liver</th>
<th>Hepatoma (rapidly growing 3683F)</th>
<th>Newborn (6 days old)</th>
<th>Regenerating (24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP dehydrogenase</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1350</td>
<td>337&lt;sup&gt;b&lt;/sup&gt;</td>
<td>495&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adenosinephosphocarboxyl-ate</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>308</td>
<td>52&lt;sup&gt;c&lt;/sup&gt;</td>
<td>113&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adenosinephosphocarboxyl-ate</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>175</td>
<td>116&lt;sup&gt;c&lt;/sup&gt;</td>
<td>115&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMP deaminase</td>
<td>100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>433</td>
<td>191&lt;sup&gt;b&lt;/sup&gt;</td>
<td>118&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GMP kinase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>121</td>
<td>125</td>
<td>96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GMP synthetase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>548</td>
<td>256&lt;sup&gt;c&lt;/sup&gt;</td>
<td>124&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Adenosine kinase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6</td>
<td>112&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Adenosine deaminase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>420</td>
<td>137&lt;sup&gt;c&lt;/sup&gt;</td>
<td>115&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Adenosine deaminase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>280</td>
<td>124&lt;sup&gt;c&lt;/sup&gt;</td>
<td>148</td>
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<tr>
<td>Formylglycinamidine ribo-nucleotide synthetase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>660</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Inosine phosphorylase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19</td>
<td>48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10</td>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uracilase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>109&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amidotransferase/xanthine oxidase</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2740</td>
<td>1364&lt;sup&gt;d&lt;/sup&gt;</td>
<td>150&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Specific activities are expressed as percentages of the normal resting adult rat liver values.
* Qualitative discriminant.
* Quantitative discriminant.

---

markedly elevated; these pools were unchanged in other tumors. The sarcoma was also distinguished from other tumors by increased GMP kinase activity which was unaltered in other neoplasms and by the decreased activities of adenosylsuccinate lyase and AMP deaminase which were markedly increased in liver and kidney tumors in the rat. Pyruvate kinase activity was decreased to 35% in sarcoma, but it was increased in all other types of tumors examined (102).

Integrated Enzymic and Metabolic Program in Neoplasia and Chemotherapeutic Targets. For the rational design of anticancer chemotherapy, it is essential to identify enzymic, metabolic, and nucleotide targets in cancer cells that are consistent elements in the biochemical commitment to neoplastic replication. However, it has been claimed that there were no biochemical differences between normal and cancer cells and that if there were any differences they were merely quantitative, not qualitative. These largely uninformed claims have been superseded by the striking advances in the elucidation of the enzymology, isozyme shift, and nucleotide pattern of cancer cells in the past 20 years [overviews were presented (8, 12, 48, 62, 89, 91–93, 95, 97, 98, 100, 114, 127, 128)]. It is informative to summarize the most relevant quantitative and qualitative differences between cancer cells and their homologous normal counterparts (Tables 6 and 7).

We have identified 43 enzymes that are transformation linked (activities altered in all cancer cells), and 26 of these are also progression linked (activities gradually altered with tumor malignancy) (Table 6). The progression-linked alterations in the activities of key enzymes and metabolic pathways in the pools of metabolites and nucleotides are summarized in Table 7. Over 70 biochemical markers have been pinpointed, most of them in this laboratory, as stringently linked quantitative correlates of neoplastic progression.

The qualitative changes, the isozyme shift, are expressed in the gradual decrease in the activity of liver-specific, regulatory, high-Km isozymes and the increase in activity of the non-liver-type, low-Km isozymes (19, 36, 48, 93, 97, 98, 127, 128).

Selective Advantages of Pleiotropic Gene Conservation and Targets of Chemotherapy. In all metabolic areas examined in hepatomas, an imbalance was revealed in the reciprocal regulation of the activities of the opposing key enzymes and synthetic and catabolic pathways. In pyrimidine metabolism, there was an increase in activities of the synthetic enzymes and a decrease in those of the catabolic ones, along with an elevation in the pools of CTP and of the dNTPs. In purine metabolism, the reprogramming of gene expression resulted in an increased potential to produce IMP, and particularly GMP, GDP, and GTP, and a decreased capacity to degrade purines. The alterations in carbohydrate metabolism, discussed elsewhere (100, 128), provide increased activities of the key enzymes of glycolysis and pentose phosphate production with a decrease in those of the key glucogenetic enzymes and gluconeogenesis. The alteration resulted in a higher capacity to glycolyze and through the increased activities of glucose-6-phosphate dehydrogenase, transaldolase, and PRPP synthetase to produce pentose phosphates and PRPP (19, 28, 29, 53, 67, 79, 84, 93, 101, 103, 105, 106, 115, 116, 121, 124, 128). The enzymic and metabolic imbalance in pyrimidine, purine, and carbohydrate metabolism confers selective reproductive advantages to cancer cells. These
advantages, although short term, result in a differential survival and reproduction of the neoplastic gene pool in the host.

The integrated enzymic imbalance in the cancer cells suggests that a number of genes involved in the production and regulation of opposing key enzymes of carbohydrate, pentose phosphate, purine, pyrimidine, and other strategic metabolic pathways are closely coadapted during evolution. Thus, the pleiotropic programming of gene expression may represent the program of one, or a complex, of master genes and integrative genomic elements (125), since the isozyme pattern of key enzymes was also stringently linked with the commitment of the cancer cells to transformation and progression. Until it becomes feasible to use direct gene therapy, the most relevant components in the neoplastic phenotype of the biochemical commitment to replication, i.e., the key enzymes, should be prime targets of selective anticancer chemotherapy.

## Design of Enzyme Pattern-targeted Chemotherapy

The design of chemotherapy that is directed against the enzyme pattern of the neoplasia is based on the following considerations: (a) the enzymic and metabolic imbalance provides a selective reproductive advantage for the cancer cells; (b) by pinpointing the biochemical alterations that characterize the commitment of cancer cells for replication, we can identify targets for anticancer drug treatment. Such biochemical targets are the transformation- and progression-linked increased activities of key enzymes and pools of nucleotides and the high metabolic requirements revealed in depleted concentrations of strategic metabolites and precursors, or amino acids; (c) it is postulated that the selectivity of the drugs against cancer cells will be the higher the more extensive is the biochemical difference from normal cells, because it is assumed that the marked metabolic imbalance reveals an increased dependence on the enzyme or the process involved; (d) because of the commitment to replication and utilization of precursors and decreased responsiveness and adaptability of the neoplastic cells to regulatory signals, the amplified and stringently linked enzymatic and metabolic pattern in neoplasia is more vulnerable to drug-induced perturbations than that of the normal tissue which has a wider range of adaptability, repair, and recovery.

Selective toxicity is based on the following criteria: Criterion 1, in the tumors there should be transformation-linked increases (a) in activities of key enzymes, (b) in the pools of strategic nucleotides and deoxynucleotides, (c) in the influx of precursors (nucleosides, deoxynucleosides, amino acids, glucose, free fatty acids) and/or a decreased concentration of strategic amino acids (glutamine) or coenzymes (NADP, NADPH); Criterion 2, enhanced selectivity is introduced by designing drug combinations to exploit the progression-linked pattern of enzymic and metabolic changes in the tumor that have conferred reproductive advantages to the cancer cells. If the metabolic alteration was important and stringently linked with the malignant replication, the tumor cells would depend on it more intensively and the perturbation of the biochemistry should be far more disruptive to the cancer cells than to normal ones. Criterion 3, in tumors that retained a segment of gene expression characteristic of the tissue of origin, drug combinations that are selectively synergistic

### Table 7

<table>
<thead>
<tr>
<th>Nucleic acid metabolism</th>
<th>Carbohydrate metabolism</th>
<th>Protein and amino acid metabolism</th>
<th>Other metabolic areas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimidine, RNA, and DNA synthesis (increased)</td>
<td>Glucose catabolism (increased)</td>
<td>Protein synthesis (increased)</td>
<td>Membrane cAMP metabolism</td>
</tr>
<tr>
<td>Carbamoyl-phosphate synthetase II</td>
<td>Hexokinase</td>
<td>Amino acid incorporation into protein</td>
<td>cAMP phosphodiesterase (increased)</td>
</tr>
<tr>
<td>Aspartate carbamoyltransferase</td>
<td>Pyruvate kinase</td>
<td>Activity of postmicrosomal protein-synthesizing system</td>
<td>Adenylate cyclase (decreased)</td>
</tr>
<tr>
<td>Dihydroorotase</td>
<td>Glucose synthesis (decreased)</td>
<td>Glutamate synthetase</td>
<td>Polyamine synthesis (increased)</td>
</tr>
<tr>
<td>CTP synthetase</td>
<td>Glucose-6-phosphatase</td>
<td>Glutamate dehydrogenase</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>Ribonucleotide reductase</td>
<td>Fructose-1,6-diphosphatase</td>
<td>Glutamate-oxaloacetate transaminase</td>
<td>Polyamine synthesis (decreased)</td>
</tr>
<tr>
<td>Thymidylate synthetase</td>
<td>Phosphoenolpyruvate carboxylase</td>
<td>Tryptophan pyrrolase</td>
<td>S-Adenosylmethionine synthetase</td>
</tr>
<tr>
<td>Deoxyctydylate deaminase</td>
<td>Pyruvate carboxylase</td>
<td>Serotonin deaminase</td>
<td>Ketone body utilization (increased)</td>
</tr>
<tr>
<td>Uridine-cytidine kinase</td>
<td>DNA polymerase</td>
<td>NADPH</td>
<td>Succinyl-CoA:acetacetyl-CoA transferase</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>Specific phosphorylating enzymes (decreased)</td>
<td>5-Hydroxytrypophan decarboxylase</td>
<td>Lipid metabolism (decreased)</td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>Fructose metabolism (decreased)</td>
<td>Enzymes degrading amino acids (increased)</td>
<td>α-Glycoerophosphate dehydrogenase</td>
</tr>
<tr>
<td>RNA polymerases</td>
<td>Thick kinase</td>
<td>Glutaminase</td>
<td>Hydroxymethylglutaryl-CoA synthase</td>
</tr>
<tr>
<td>tRNA methylase</td>
<td>Aldolase</td>
<td>Urea cycle (decreased)</td>
<td>NADP content (decreased)</td>
</tr>
<tr>
<td>CTP concentration</td>
<td>Isozyme shift (decreased)</td>
<td>Urea cycle transferase</td>
<td>NADP</td>
</tr>
<tr>
<td>dCTP concentration</td>
<td>High-Km isozymes</td>
<td>Ornithine carbamoyltransferase</td>
<td>NADPH</td>
</tr>
<tr>
<td>dTTP concentration</td>
<td>Low-Km isozymes</td>
<td>* cAMP, cyclic adenosine 3':5'-monophosphate.</td>
<td></td>
</tr>
<tr>
<td>Pyrimidine catabolism (decreased)</td>
<td>Formylglycinamidme ribonudeotide synthetase</td>
<td>Glutamate-oxaloacetate synthetase</td>
<td></td>
</tr>
<tr>
<td>Dihydrooracil dehydrogenase</td>
<td>IMP dehydrogenase</td>
<td>PRPP synthetase</td>
<td></td>
</tr>
<tr>
<td>Purine synthesis (increased)</td>
<td>GMP synthetase</td>
<td>Ribose 5-phosphate utilization (increased)</td>
<td></td>
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<tr>
<td>Formylglycinamidme ribonucleotide synthetase</td>
<td>AMP deaminase</td>
<td></td>
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<tr>
<td>IMP dehydrogenase</td>
<td>GMP concentration</td>
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<tr>
<td>dGMP concentration</td>
<td>dGTP concentration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dATP concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrimidine catabolism (decreased)</td>
<td>Adenylate kinase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* cAMP, cyclic adenosine 3':5'-monophosphate.
in cells containing the enzyme patterns characteristic both of proliferation or neoplasia and of the tissue of origin of the tumor can be utilized (37, 39). Evidence was published from this laboratory showing the feasibility of this approach (37, 39, 55–57, 118, 120).

Targeting Chemotherapy against Glutamine-utilizing Enzymes in Rat Hepatoma

The principles developed for an enzyme pattern-targeted chemotherapy have been tested in a series of investigations in this laboratory (4, 5, 37, 39, 55–57, 118, 120). One such program was designed on the basis of the imbalance we discovered in glutamine metabolism. This approach is now illustrated in some detail.

To justify launching chemotherapy against an altered enzyme pattern in cancer cells, several criteria should be fulfilled. To design anti-glutamine-targeted chemotherapy, the following biochemical alterations should be demonstrated in the cancer cell.

The Drug Treatment Is Targeted against an Essential Part of the Neoplastic Biochemical Program. That a change in glutamine metabolism is an essential aspect of the biochemical program of neoplastic cells is suggested by the observation that the increases in the activities of enzymes involved in glutamine utilization were strongly conserved in different types of animal and human neoplasms. The specific activities of glutamine-utilizing enzymes in purine synthesis (amidophosphoribosyltransferase and GMP synthetase) and in pyrimidine synthesis (carbamoyl-phosphate synthetase II and CTP synthetase) increased in the series of rat hepatomas (120). The elevations of amidotransferase activity were transformation linked, whereas those of the activities of the other 3 enzymes were both transformation and progression linked. The activities of the 4 enzymes also increased in renal cell carcinoma and sarcoma in rat and in human renal cell carcinoma and colon carcinoma (120) (Table 8).

Increased Enzymic Activities Are Reflected in the Pattern of the Products and in the Metabolic Pathways. The impact of increased enzymic activities should be revealed in alterations in the concentrations of strategic ribonucleotides. Freeze-clamp studies determining the in vivo nucleotide pools in transplanted hepatoma 3924A showed that the concentration of CTP (the product of CTP synthetase) was increased 4-fold and that of GMP (the product of GMP synthetase) was elevated 3.4-fold over the concentration of the normal liver (34).

Impact of the Altered Enzymic Pattern Is Expressed in the Pools of dNTP, the Immediate Precursors of DNA. The increased activities of glutamine-utilizing enzymes and the enlargement in the pools of the nucleotides produced are reflected in the amplification of dNTP pools. The freeze-clamp studies in hepatoma 3924A indicated that the pools of dNTPs (the products of ribonucleotide reductase) were markedly enlarged with the concentrations of dGTP, dATP, dTTP, and dCTP increasing 4.8-, 17.7-, 12.1-, and 7.9-fold, respectively (34).

Pool of an Essential Metabolic Precursor in Cancer Cells, L-Glutamine, Is Depleted. This criterion requires evidence that this is a biologically significant depletion in tumor cells in the concentration of relevant metabolic precursors as compared to the levels found in normal tissues. In the freeze-clamp studies, the in vivo concentration of L-glutamine in hepatoma 3924A was 9-fold lower (0.5 mw) than that in liver (4.5 mw) and lower than in any other rat tissues (2 to 5 mw). The low glutamine concentra-

### Table 8

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Amido-transfase</th>
<th>GMP synthetase</th>
<th>Carbamoyl-phosphate synthetase II</th>
<th>CTP synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat hepatoma 3683F</td>
<td>212</td>
<td>548</td>
<td>946</td>
<td>1061</td>
</tr>
<tr>
<td>Rat renal cell carcinoma, MK-3</td>
<td>220</td>
<td>297</td>
<td>495</td>
<td>430</td>
</tr>
<tr>
<td>Rat sarcoma</td>
<td>1345</td>
<td>218</td>
<td>1805</td>
<td>7768</td>
</tr>
<tr>
<td>Human renal cell carcinoma</td>
<td>158</td>
<td>167</td>
<td>180</td>
<td>181</td>
</tr>
<tr>
<td>Human colon tumor</td>
<td>183</td>
<td>329</td>
<td>314</td>
<td></td>
</tr>
</tbody>
</table>

Chart 19. Enzymic imbalance in glutamine synthesis, catabolism, and utilization in rapidly growing hepatoma 3924A. GMP synth.; GMP synthetase; CTP synth.; CTP synthetase; carbamoyl-phosphate synthetase II; FGAR AT, phosphoribosylformylglycinamidine synthetase; PRPP AT, amidophosphoribosyltransferase (amidotransferase); glutamine synth.; glutamine synthetase.

Chart 19: Enzymic imbalance in glutamine synthesis, catabolism, and utilization in rapidly growing hepatoma 3924A. GMP synth. GMP synthetase; CTP synth., CTP synthetase; carbamoyl-phosphate synthetase II; FGAR AT, phosphoribosylformylglycinamidine synthetase; PRPP AT, amidophosphoribosyltransferase (amidotransferase); glutamine synth., glutamine synthetase.

Drug selectivity against hepatoma 3924A is anticipated because, through the higher glutamine-utilizing enzymic activities and the enlarged pools of GTP, CTP, and dNTPs, the cancer cells are committed to utilizing these biosynthetic capacities. The low concentration of L-glutamine in this hepatoma should provide...
Lower protection from the antiglutamine agent for the active centers of the key enzymes of glutamine utilization. In the resting liver, with its lower requirement for GTP, CTP, and the dNTP pools, and for the activities of the glutamine-utilizing enzymes, and because the higher levels of glutamine might protect these enzymes, decreased sensitivity to the antiglutamine agent is expected.

**Enzyme Pattern-targeted Action of the Antiglutamine Agent, Acivicin**

The antiglutamine agent acivicin, L-αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, was isolated from Streptomyces sviceus and exhibited antitumor activity against L1210 and P388 leukemias in mouse and human lung and in breast xenografts in athymic mice (26). Neil et al. (64, 65) showed that in L1210 cell culture acivicin blocked DNA synthesis and arrested cell cycle progression in early S phase; glutamine protected the cells. Jayaram et al. (40) demonstrated that acivicin competitively inhibited the activities of various bacterial and mammalian enzymes that catalyze the transfer of the amido group of L-glutamine. Further work showed that acivicin was a competitive inhibitor of purified rat liver CTP synthetase (65). We tested the approaches of enzyme pattern-targeted chemotherapy by elucidating the action of acivicin in tissue culture of hepatoma cells and in vivo in rats carrying transplantable hepatoma 3924A (17, 57, 120, 134).

**Action of Acivicin in Hepatoma 3924A Tissue Culture Cells and Protection by Nucleosides.** Acivicin, on incubation for 7 days, killed hepatoma cells with an LD₅₀ of 1.4 μM as measured by clonogenic assay (57). When hepatoma cells were grown in log phase, 72-hr incubation with acivicin (100 μM) resulted in 55% growth inhibition, as measured by cell counts. Addition of pyrimidine or purine nucleosides (cytidine, deoxycytidine, guanosine) singly to the medium yielded no protection. The 3 nucleosides together provided complete protection against acivicin (57). These results supported the idea that the increased glutamine-utilizing enzyme activities were essential for the hepatoma cells and that both purine and pyrimidine synthesis were involved in the program of commitment to neoplastic growth. The protection by nucleosides implied the presence of appropriate activities of the salvage enzymes, uridine-cytidine and deoxycytidine kinases, and hypoxanthine-guanine phosphoribosyltransferase. The result confirmed our conclusions, drawn from enzymic studies outlined earlier in this paper, that the salvage pathways have a vital role in determining the anticancer action of antimetabolites on the de novo pathways and that blockers of salvage enzymes or of nucleoside transport should be utilized in combination chemotherapy.

**Action of Acivicin on Pools of Ribonucleotide Triphosphate and dNTP in Hepatoma Cells in Tissue Culture.** Treatment with acivicin for 16 hr decreased the concentrations of GTP and CTP to 62 and 46%, respectively, and accumulated that of UTP to 152% of controls; ATP level was not changed. The pools of dGTP, dCTP, and dTTP decreased to 64, 40, and 53% controls, with no change in the dATP pools (Table 9). The results were compatible with an explanation that acivicin inhibited the activity of GMP synthetase, resulting in depression of GTP. Furthermore, the drug inhibited CTP synthetase, yielding a decrease in concentration of the product, CTP, and an accumulation of the substrate, UTP. The resulting decrease in the nucleoside diphosphate content should account for the depletion of the pools of dGTP, dCTP, and dTTP.

**Table 9**

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>Control: no drug added (mmol/10⁹ cells)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>3373 ± 98*</td>
<td>87%</td>
</tr>
<tr>
<td>GTP</td>
<td>741 ± 8*</td>
<td>62%</td>
</tr>
<tr>
<td>CTP</td>
<td>904 ± 64*</td>
<td>45%</td>
</tr>
<tr>
<td>UTP</td>
<td>1654 ± 107*</td>
<td>152%</td>
</tr>
<tr>
<td>dATP</td>
<td>157 ± 4*</td>
<td>124%</td>
</tr>
<tr>
<td>dGTP</td>
<td>104 ± 6*</td>
<td>64%</td>
</tr>
<tr>
<td>dCTP</td>
<td>147 ± 15*</td>
<td>40%</td>
</tr>
<tr>
<td>dTTP</td>
<td>250 ± 27*</td>
<td>53%</td>
</tr>
</tbody>
</table>

*Significantly different from control (p < 0.05).

**Action of Acivicin on Glutamine-utilizing Enzymes in Hepatoma in Vitro.** Purified CTP synthetase from hepatoma 3924A was competitively inhibited by acivicin at Kᵢ = 1 μM (57). The activity of GMP synthetase in the crude extracts was inhibited by acivicin at Kᵢ = 7 μM; this was mixed inhibition. The other 2 glutamine-utilizing enzymes were competitively inhibited by acivicin in hepatoma extracts (Table 10). Aoki et al. (2) reported that the activity of purified carbamoyl-phosphate synthetase II from rat liver and hepatoma 3924A was competitively inhibited by acivicin at Kᵢ = 7 μM. The activity of amidophosphoribosyltransferase, studied in crude extracts of liver and hepatoma, was inhibited at Kᵢ = 5 mM (120). These observations are in good agreement with those of Jayaram et al. (40), who showed in other tissues that a series of glutamine-utilizing enzymes were competitively inhibited by acivicin.

**Inactivation by Acivicin of Glutamine-utilizing Enzymes in Vitro and in Vivo and Selectivity.** Our further studies threw novel light on the action of acivicin. The inhibition of the enzymes was not reversible in vitro, yet this should have been so in case of competitive inhibition unless we were dealing with a very tightly binding inhibitor. On in vivo injection of acivicin, the activities of the glutamine-utilizing enzymes markedly declined within minutes, and the decreased enzyme activities remained low for many hr after acivicin disappeared from the blood stream (17, 120). These in vivo studies, to be outlined below, were not compatible with a simple competitive inhibition and implied the operation of another mechanism.

Investigations in vitro revealed that the purified or crude enzymes were rapidly inactivated by addition of acivicin, and no method that we used could reactivate them. Acivicin in vitro selectively inactivated the glutamine-dependent activity of carbamoyl-phosphate synthetase II from partially purified extracts of hepatoma and liver, with an inactivation constant, Kᵢ, of 90 μM, and a minimum inactivation half-time, Tᵢ, of 0.7 min. L- Glutamine (1 mM) protected the enzyme from inactivation by 10 μM acivicin. The ammonia-utilizing and aspartate carbamoyltransferase activities of the enzyme complex were not inhibited by acivicin. The inhibited synthetase II activity was not restored by (a) purification of the enzyme involving extensive dilutions (1:1000) at 4 steps, (b) gel filtration of the enzyme-acivicin complex, (c) addition of a high concentration of L-glutamine (50 mM) to the assay mixture, or (d) freezing (in liquid nitrogen) and thawing the complex (2).

In rats carrying transplant hepatoma 3924A, injection of acivicin (25 mg/kg i.p.) caused a strikingly rapid decrease in the
activities of the glutamine-utilizing enzymes in the hepatoma. Significant declines were detected at 10 min after injection, and by 6 hr amidotransferase, CTP synthetase, and carbamoyl-phosphoribosyltransferase II activities decreased to 34, 4, and 6%, respectively, of the controls. Aspartate carbamoyltransferase activity did not change (17, 120) (Chart 20). With an acivicin dose of 5 mg/kg, GMP synthetase activity in the hepatoma decreased to 9% of the control at 6 hr after injection. All enzymic activities of 5 mg/kg, GMP synthetase activity in the hepatoma decreased. With an acivicin dose of 5 mg/kg, GMP synthetase activity in the hepatoma decreased to 9% of the control at 6 hr after injection. All enzymic activities bottomed out at these low levels for 12 hr after injection and then slowly started to return toward normal range by 48 to 72 hr (Table 11). Enzymic activities in the host liver also decreased, but these changes were less pronounced than those in the hepatoma; the pattern of return was similar to that in the hepatoma.

The rapid in vitro and in vivo inactivation of the 4 glutamine-utilizing enzymes (Table 11) may be explained by the observation that in bacterial systems acivicin acted as an active site-directed affinity analogue of L-glutamine (86). We postulated that the rapid action of acivicin in vivo was due to alkylation of the active center; thus, the L-glutamine concentration of the tissues might be a major determining factor in acivicin toxicity.

Action of Acivicin in Vivo on Ribonucleotide Pools in Hepatoma 3924A. These experiments were designed to elucidate whether and to what extent the changes in enzymic activities were reflected in the concentrations of the relevant nucleotides. At various time periods after acivicin injection, groups of rats bearing hepatoma 3924A were anesthetized, and tumors and host livers were freeze-clamped (Table 12). In the hepatomas, ATP and UTP concentrations remained unchanged. The GTP pool decreased to 56% of control 2 hr after injection, and the lowest value was reached at 6 hr (32%); the pool was still significantly decreased at 24 hr, returning to normal range by 48 and 72 hr. The CTP concentration decreased to 46% at 30 min after injection, and then reached the lowest level (2%) at 2 hr. CTP pool remained at less than 20% for 24 hr, and returned to normal range at 48 and 72 hr after injection. In the host liver, the concentrations of ATP and UTP did not change, and those of GTP and CTP decreased in the same pattern as in the hepatomas, but to a minor degree (17, 120). The differential selectivity of acivicin impact on hepatoma and liver may be due, in part at least, to the higher glutamine content of the host liver.

It is important that the decline in enzymic activities preceded that in the pools of GTP and CTP (Chart 21). There was a differential sensitivity to acivicin, with CTP synthetase and GMP synthetase being the most, and amidophosphoribosyltransferase the least, sensitive to the antiguamine agent (Table 11). The K for acivicin for these enzymes determined in vitro roughly correlated with their in vivo sensitivity to this drug (Table 10).

The mechanism of competitive inhibition could not play an important role in the biological action of acivicin, since the inhibitions were not reversible. The decrease in enzymic activities

### Table 10

**Inhibition by acivicin of activities of glutamine-utilizing enzymes in hepatoma 3924A**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Extract</th>
<th>Glutamine concentration in standard assay (nmol/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glutamate K&lt;sub&gt;r&lt;/sub&gt; (mM)</td>
</tr>
<tr>
<td>Amidotransferase</td>
<td>Crude supernatant</td>
<td>2.3</td>
</tr>
<tr>
<td>GMP synthetase</td>
<td>Crude supernatant</td>
<td>0.2</td>
</tr>
<tr>
<td>CTP synthetase</td>
<td>Purified preparation</td>
<td>0.5</td>
</tr>
<tr>
<td>Carbamoyl-phosphatase</td>
<td>Purified preparation</td>
<td>0.017</td>
</tr>
</tbody>
</table>

### Table 11

**In vivo effect of acivicin on enzyme activities in hepatoma 3924A**

<table>
<thead>
<tr>
<th>Time after acivicin injection (hr)</th>
<th>Amidotransferase</th>
<th>GMP synthetase</th>
<th>CTP synthetase</th>
<th>Carbamoyl-phosphoribosyltransferase I (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>142 ± 4.4a</td>
<td>55.9 ± 1.6</td>
<td>56.5 ± 2.6</td>
<td>82.0 ± 8.6</td>
</tr>
<tr>
<td>10 min</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5 hr</td>
<td>87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72</td>
</tr>
<tr>
<td>1 hr</td>
<td>56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 hr</td>
<td>32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 hr</td>
<td>21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12 hr</td>
<td>34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 hr</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>48 hr</td>
<td>46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>72 hr</td>
<td>74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.E. of 4 or more rats in each group.
<sup>b</sup> Significantly different from controls (p < 0.05).

### Table 12

**Effect of acivicin on nucleoside triphosphate pools in hepatoma 3924A**

<table>
<thead>
<tr>
<th>Time after acivicin injection (hr)</th>
<th>ATP</th>
<th>GTP</th>
<th>CTP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10 min</td>
<td>100</td>
<td>93</td>
<td>120</td>
<td>127</td>
</tr>
<tr>
<td>0.5 hr</td>
<td>85</td>
<td>76&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>123</td>
</tr>
<tr>
<td>1 hr</td>
<td>70</td>
<td>69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104</td>
</tr>
<tr>
<td>2 hr</td>
<td>70</td>
<td>32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103</td>
</tr>
<tr>
<td>6 hr</td>
<td>77</td>
<td>32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>122</td>
</tr>
<tr>
<td>12 hr</td>
<td>64</td>
<td>42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>106</td>
</tr>
<tr>
<td>24 hr</td>
<td>65</td>
<td>57</td>
<td>19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77</td>
</tr>
<tr>
<td>48 hr</td>
<td>117</td>
<td>118</td>
<td>89</td>
<td>98</td>
</tr>
<tr>
<td>72 hr</td>
<td>108</td>
<td>130</td>
<td>89</td>
<td>120</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.E. of 4 or more rats in each group.
<sup>b</sup> Significantly different from controls (p < 0.05).
was strikingly rapid, with significant declines detected at 10 min after injection. The decreased enzymic activities observed after in vivo injection of acivicin could not be reactivated in vitro by incubation with high concentrations of L-glutamine or other methods used. In the case of carbamoyl-phosphate synthetase II, activity could not be restored by purifying the enzyme through the steps that should have removed acivicin, unless it was very tightly bound to the active center or the enzyme was inactivated. The results indicated to us that inactivation of the enzyme by acivicin was carried out by affinity labeling of the glutamine-binding site (86). The action of acivicin was not limited to hepatoma, because similar in vivo inactivation of the synthetase II was observed in a chemically induced transplantable sarcoma carried in the rat. The activity of aspartate carbamoyltransferase was unaffected (120).

Combination Chemotherapy of Acivicin with Actinomycin in Hepatoma 3924A in Vitro and in Vivo. The 4 ribonucleoside triphosphates are the substrates for RNA polymerase for synthesis of RNA. RNA polymerase was a promising target because the activity was markedly increased in hepatoma 3924A (18). Since the acivicin action was expressed in depletion of GTP and CTP pools, it was expected that actinomycin which inhibited RNA polymerase to utilize UTP and CTP would yield a synergistic response (see Chart 21). This rationale led us to study the effects of acivicin and actinomycin separately and together in the culture medium of hepatoma 3924A; cell kill was measured by colony counts. The LD50 values for acivicin and actinomycin were 1 and 0.01 µM, respectively, and the 2 drugs together were synergistic in killing hepatoma cells (120).

In similar studies in hepatoma cells in culture, acivicin alone decreased GTP and CTP concentrations, whereas actinomycin alone caused a 5-fold increase in CTP pools and a 1.5-fold rise in UTP concentration. When acivicin and actinomycin were added to the cells together, the decrease in GTP and CTP pools caused by acivicin was overcome by actinomycin which raised GTP and CTP pools 2- and 4-fold (120). These observations may explain, in part at least, the synergistic cytotoxic action of actinomycin and acivicin in hepatoma cells.

A possible in vivo synergistic action of acivicin and actinomycin was tested in rats inoculated with hepatoma 3924A tumor fragments. Groups of rats were treated with acivicin, or with actinomycin, and with a combination of the 2 drugs. Treatment was started 24 hr after the tumor cells were inoculated, and volumes were calculated by measuring tumor diameters. The results showed that combination chemotherapy yielded a synergistic inhibition of tumor growth, measured 14 days after tumor inoculation (Table 13) (120).

Enzymic and Nucleotide Targets and Biochemical Basis of Synergistic Action of Acivicin and Actinomycin: Role of Salvage Pathways. Purine- and pyrimidine-biosynthetic processes from de novo and salvage pathways culminate in production of ATP, GTP, UTP, and CTP. These nucleotides are substrates for RNA polymerase, which synthesizes RNA. Acivicin selectively decreased concentrations of GTP and CTP with little change in those of ATP and UTP. These observations and a report that RNA polymerase activity was markedly increased in hepatoma 3924A (18) led us to design experiments using actinomycin which selectively blocked the utilization of GTP and CTP by RNA polymerase (32) (Chart 22).

It was remarkable that, although acivicin injection markedly decreased the activities of the key enzymes, amidophosphoribosyltransferase and GMP synthetase, only the GTP levels decreased, but ATP pools did not. In pyrimidine biosynthesis, the activities of carbamoyl-phosphate synthetase II and CTP synthetase were inhibited, but this was expressed only in the depletion of CTP pools, and UTP concentrations were unchanged. The protection of ATP and UTP pools was tentatively attributed to the contribution of the pyrimidine and purine salvage pathways. This hypothesis was tested in the following experiments.

In tissue culture studies, we showed that in hepatoma 3924A cells the salvage nucleosides, uridine, deoxyribosyl cytosine, and guanine together were able to protect the cancer cells from the action of acivicin (57). This observation indicated the presence in the cells of adequate activities of the salvage enzymes, uridine and deoxycytidine kinases and GRP.

In further investigations, we compared in liver and hepatomas the activities of the key enzymes of de novo and salvage pathways of pyrimidine and purine synthesis. The results (outlined above) indicated that in the rapidly growing hepatoma 3924A the salvage enzymes were present in high activities. Further important determinants include the concentrations of salvage precursors, substrates, and cofactors (uracil, uridine, cytidine, deoxycytidine, thymidine, adenosine, hypoxanthine, guanine, PRPP, ATP, etc.), all of which impact on the contributions of the de novo and salvage pathways.

Thus, the inhibition of carbamoyl-phosphate synthetase II was counterbalanced by the high uridine kinase activity in the hepatoma, which was sufficient to maintain the UTP pool at the prevailing tissue uridine level. Similarly, the inhibition of amidophosphoribosyltransferase activity was counterbalanced by the high APRT activity which at the tissue adenine concentrations was able to maintain the ATP pool. The decrease in GTP concentration was attributed to the marked inhibition of GMP synthetase (CTP S) activity, and CTP concentration.
Cytotoxic Action of Dipyridamole in Hepatoma 3924A Cells.
Logistically growing hepatoma cells were incubated with dipyridamole, and survival was determined by clonogenic assay. In treatment schedules of 1 or 7 days, LD₅₀ values were 87 and 20 μM, respectively. These dipyridamole concentrations were higher than those required for the 50% inhibitory concentrations for blocking nucleoside incorporations which were 0.2 to 0.5 μM for uridine, cytidine, thymidine, and guanosine. Since we demonstrated that dipyridamole was effective in killing hepatoma cells, we decided to investigate the biochemical mechanism of action.

Dipyridamole Action on Cell Survival and DNA, RNA, and Protein Synthesis. In dose-response studies, 10 to 75 μM dipyridamole inhibited incorporation of uridine and thymidine but not that of formate, glycine, and leucine. Since dipyridamole under these conditions inhibited the incorporation of pyrimidine and purine nucleosides, its blocking of RNA and DNA synthesis may be attributed, in part at least, to inhibition of transport of salvage precursors.

With dipyridamole concentrations of 60 to 100 μM, the pools of ATP, GTP, CTP, and UTP decreased to 27, 50, 19, and 23%, respectively, and 76% of the hepatoma cells were killed. This raised the possibility that dipyridamole interfered with the biochemical commitment of hepatoma cells by depleting the pools of the 4 ribonucleotides.

Synergistic Action of Dipyridamole and Acivicin on Hepatoma Cells. When acivicin or dipyridamole was incubated with hepatoma cells, survivals of 64 and 65%, respectively, were observed. Combination of the 2 drugs yielded 11% survival, indicating synergism. These results support our idea that combination chemotherapy with an antimetabolite of the de novo pathways (acivicin) and an inhibitor of the salvage pathways (dipyridamole) might yield synergistic anticancer cytotoxic action. In vivo administration of acivicin, dipyridamole, and the 2 drugs together in rats bearing s.c. transplanted hepatoma 3924A showed that acivicin decreased mainly the GTP and CTP pools, whereas dipyridamole depleted the concentrations of all 4 ribonucleotides. Combination chemotherapy with the 2 drugs yielded summation, decreasing ATP, GTP, CTP, and UTP levels to 23, 14, 15, and 29%, respectively, of the control. The decline in UTP concentration was a synergistic response (112).

These drugs also had a marked impact in the hepatoma on the dNTP pools. Acivicin lowered dATP, dGTP, and dCTP pools without affecting dTTP concentration. Dipyridamole depleted dGTP and dCTP pools to 52 and 22%, respectively. Acivicin and dipyridamole in combination yielded a summation, decreasing the pools of dATP, dGTP, dCTP, and dTTP to 64, 34, 17, and 44%, respectively, of the controls (112). The attacking points of acivicin and dipyridamole in pyrimidine biosynthesis are summarized in Chart 23.

The results indicate that a protocol of combination chemotherapy of effective antimetabolites (acivicin, tiazofurin, and others) and an inhibitor of nucleoside transport (dipyridamole) is productive, particularly because the antimetabolites are in Phase II clinical trials and dipyridamole has been used in clinical practice (for different therapeutic objectives) for many years. Investigations with these drugs throw light on their mechanism of action and on the relative contributions of de novo and salvage pathways in normal and neoplastic tissues in animals and in humans.

Effect of the Transport Inhibitor, Dipyridamole, on Hepatoma 3924A Cells
Experimental and clinical investigations indicated that inhibition of the de novo synthetic pathways of pyrimidine and purine metabolism by antimetabolites failed to yield lasting remissions in neoplastic diseases. Our studies on experimental and primary human neoplasms showed that along with increased activities of key enzymes of nucleic acid biosynthesis there also were elevations in the activity of the salvage enzymes of pyrimidine synthesis and maintenance of the high activities of purine salvage enzymes. From the studies of others and those presented in this paper, there is evidence that nucleosides can protect and rescue cancer cells from the cytotoxic action of inhibitors of de novo nucleic acid biosynthesis. Our chain of reasoning led us to determine whether blockers of nucleoside transport would provide synergistic anticancer action (112, 134).

Effect of Dipyridamole on Nucleoside Incorporation in Hepatoma 3924A Cells. Recently, we showed that dipyridamole in concentrations of 10⁻⁶ to 10⁻⁷ effectively blocked incorporation of purine and pyrimidine nucleosides into macromolecules in hepatoma 3924A cells (134). Our investigations demonstrated that dipyridamole was 9- to 27-fold more effective than was nitrobenzylthioinosine; therefore, all our studies were carried out with dipyridamole, which has the added advantage that it is a licensed drug in the United States Pharmacopoeia.

Chart 22. Enzymic and nucleotide targets in the synergistic action of acivicin and actinomycin and the role of salvage pathways. AT, amidotransferase; GMP synth., GMP synthetase; CTP synth., CTP synthetase; Actino. D, actinomycin D; C-P synth. II, carbamoyl-phosphate synthetase II.

Effect of Dipyridamole on Nucleoside Incorporation in Hepatoma 3924A Cells
The drug targeting approach that we proposed on basis of enzymic imbalance is a productive one. Robins wrote, "The postulation of Weber that IMP dehydrogenase is a key enzyme in neoplasia and therefore a sensitive target for cancer chemotherapy has received considerable experimental support [98]. It would appear that the search for potential inhibitors of IMP dehydrogenase will continue to be a fruitful area for the design and synthesis of potent antitumor agents [75]." One of the components synthesized, tiazofurin, proved to be a good inhibitor of IMP dehydrogenase, and in a series of brilliant studies from the laboratories of Robins, Johns, Cooney, Jayaram, and Glazer, it was shown that a number of tumors including the Lewis lung tumor were particularly sensitive to tiazofurin action (14, 20, 41, 42, 76).

In current work in my laboratory, in collaboration with Dr. Jayaram, we have shown that tiazofurin is a good inhibitor of the markedly increased activity of IMP dehydrogenase in hepatoma 3924A. Tiazofurin killed hepatoma cells in tissue culture with an LD50 of 5 μM. In animals inoculated with hepatoma 3924A tiazofurin, as a single drug (150 mg/kg i.p. daily for 5 days), caused an 85% inhibition of tumor growth as determined 14 days after tumor transplantation. Our investigations demonstrated that a single in vivo injection of tiazofurin (200 mg/kg) caused a rapid decline in the activity of IMP dehydrogenase in the hepatoma and a concurrent 15-fold rise in the concentration of IMP. Following the decline in IMP dehydrogenase activity, the pools of GMP and GTP markedly declined, and the concentration of dGTP was depleted to lower than 20%. All parameters returned to normal after one single injection by 24 to 48 hr; however, the dGTP pool remained depressed for 72 hr. The marked decrease in GTP and particularly the sustained depletion in the dGTP pools may explain, in part at least, the chemotherapeutic action of tiazofurin on hepatoma 3924A (Chart 24). This is the first time that a marked therapeutic response was achieved against rapidly growing hepatoma 3924A by single drug administration.

This is drug design targeted against a key enzyme, IMP dehydrogenase, on the basis that its activity was markedly increased in this hepatoma, an example of enzyme pattern-targeted chemotherapy.

It is only a matter of time, effort, and support that through discoveries in the biochemical pharmacology of cancer cells rational therapy should be successful for all neoplastic diseases.

Conclusions

The introduction of the molecular correlation concept and the key enzyme concept and the use of biologically meaningful tumor models and control systems resulted in the discovery of an ordered pattern of enzymic and metabolic imbalance and the elucidation of the linkage with transformation and progression. With the conceptual and experimental guidance of the molecular correlation concept, we showed that the biochemical and enzymic pattern of alterations was the result of a reprogramming of gene expression that was both quantitative and qualitative and was characteristic to neoplasia, since no similar pattern of imbalance has been observed in any of the control normal, regenerating, or differentiating tissues.

Important aspects of gene logic were identified. These include demonstration of operation of reciprocal control of activities of...
opposing key enzymes and antagonistic pathways of synthesis and catabolism in pyrimidine, purine, ornithine, and carbohydrate metabolism. It was established that the extent of increase in the activities of key enzymes of pyrimidine and purine biosynthesis related to the absolute activity of the enzymes in the resting liver. Light was thrown also on qualitative alterations in gene expression. These included the isozyme shift where key regulatory enzymes, which were subject to nutritional and endocrine regulation and had a high Km, for their substrate, were replaced, particularly in the rapidly proliferating hepatomas, with an isozyme population that was less subject to nutritional and hormonal regulation and had a low Km for their substrates.

The activities of the key enzymes in purine and pyrimidine metabolism in the resting liver were low in comparison with the high activity of the enzymes of the salvage pathways. In pyrimidine metabolism, the activities of the de novo and salvage enzymes increased markedly in a transformation- and progression-linked fashion. In contrast, in purine biosynthesis only the activities of the key enzymes of de novo biosynthesis were elevated, and those of the salvage pathways changed little.

The pattern of enzymic and metabolic imbalance was present in 16 different types of neoplasms, including chemically induced, transplantable tumors in rodents, virally produced tumors in chickens, and various neoplasias in humans. Two generalizations were proposed. Since the enzymic and metabolic imbalance was present in different types of tumors, a segment of gene expression that is essential for neoplasia has been identified. The biochemical phenotype of neoplasia was independent from the carcinogen, since it was present in chemically, virally, and spontaneously induced tumors in animals and humans. Therefore, the biochemical imbalance originally discovered and documented most extensively in the spectrum of hepatomas of different growth rates was generally applicable, in part or entirely, to all tumors examined thus far. In addition, individual tumor markers have been noted for a number of neoplasms.

With these and other concepts and observations outlined above, insight was gained into strategic aspects of the biochemical commitment of cancer cells to replication. It has become possible to pinpoint the selective advantages that reprogrammed gene expression conferred to cancer cells. In turn, understanding these alterations in the enzymology and biochemistry of cancer cells made it possible to identify potentially sensitive targets for anticancer chemotherapy.

On the basis of these advances, it became feasible to design an enzyme pattern-targeted chemotherapy. In this strategy, drugs were directed against immediate targets, activities of key enzymes, to perturb the steady state of secondary targets, the pools of ribonucleotides; then it became possible to assess the effectiveness of chemotherapy by measuring the perturbation in the pools of dNTP. By carefully correlating the extent of depression of the activities of key enzymes and the pools of nucleotides with the survival of cancer cells, determined by clonogenic assays and by the volume of tumors measured in tumor-bearing animals, the response to chemotherapeutic targeting could be quantitated.

Advances have been made in showing the role of nucleosides in determining anticancer action and the chemotherapeutic use of drugs that can synergistically inhibit activities of key enzymes and the transport processes in de novo and salvage pathways.

Research in this and other laboratories in recent years demonstrated that beyond the sheer complexity and apparent diversity of the biochemistry of cancer cells it is possible to perceive the pattern that characterizes the commitment of cancer cells to replication. Inasmuch as the notion of causality and order pervades the approach I have outlined, it seems that the Ariadne thread is firmly in hand. Observations no longer need to range over a diversity of metabolic changes in cancer cells; they can now be targeted to the transformation- and progression-linked biochemical alterations which should have bearing on the rational design of selective anticancer chemotherapy.

References


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