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REGULATIONS OF ENZYMES IN ANIMALS: EFFECTS OF DEVELOPMENTAL PROCESSES, CANCER AND RADIATION

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Controls by genes and by adaptive phenomena of the patterns of enzymes in several rat tissues have been extended by systematic investigations during development, in neoplasms and after X-irradiation. New quantitative analyses of the tryptophan pyrrolase and phenylalanine hydroxylase were developed. These were used for study of the specific degradation after induction of the former enzyme, and for description of the distribution, control and function of the latter as a model for investigations in patients with phenylketonuria. Quantitative analyses of other enzymes by two different assays showed in the case of glycogen phosphorylase that relative tissue concentrations were reproducible when measured by assays with different absolute activities, and in the case of glutamine synthetase showed the existence of an unsuspected new enzyme transferring the glutamyl- group from glutamine to hydroxylamine.

Evidence was found for the existence in mammary gland of a new kind of arginase, not concerned with the urea cycle.

Patterns of enzymes quantitatively measured in a variety of tissues have been accumulated in sufficient number to demonstrate several orderly relationships among rat tissues. These include the greater chemical similarities among undifferentiated fetal tissues than among differentiated adult tissues, the greater chemical similarities among undifferentiated tumors than among their differentiated tissues of origin, and the similarities between tumors and fetal tissues. A certain chemical and behavioral orderliness is emerging among our spectra of tumors which will allow some predictions about the nature of these tumors.

Statement of Time or Effort of the Principal Investigator

During the current year of the project, the Principal Investigator, W. Eugene Knox, M. D., Senior Research Associate at the Cancer Research Institute, has spent 50% of his time devoted to the research of this project.

It is expected that during the coming year, Dr. Knox will continue to spend 50% of his time on this project.

The national survey of phenylketonuric patients completed one year ago demonstrated that early treatment with low phenylalanine diets was effective in minimizing the genetic consequence of mental retardation in this disease. Several modifications in diagnostic and treatment practices were recognized as potential improvements, and these are being cautiously introduced in the practice of several clinical centers. Further detailed classification of the cases seems to require information about the nature of the enzyme, phenylalanine hydroxylase, the inactivity of which is at the root of this metabolic error. Variants of the enzyme, and variable times of its maturation in neonatal liver, have been invoked to explain disturbances in phenylalanine metabolism of different degrees. Clinically such cases are classified in numerous ways, such as classical and atypical phenylketonuria, and as hyperphenylalanemia. Pedigree and clinical information is being accumulated in the country at large to sharpen the several categories, and a limited number of patients have been submitted to liver biopsies for direct analysis of the enzyme activity. It was apparent that investigations of the qualitative nature of the rat liver enzyme were sufficiently advanced to design a quantitative analysis of this enzyme, and that such a method should be available for studies in experimental animals as well as for the occasional patient on whom such observations might be justified.

Such a method was worked out (3) and used to describe the development, distribution and adaptive control (4) of the enzyme in rats. As with other quantitative enzyme analyses, the procedure was designed to provide assurance that the measured activity was proportional to the concentration of the enzyme in tissues, and to be a sensitive and robust assay.

The enzyme was long considered to be limited to liver. With the new, sensitive method for determining the phenylalanine hydroxylase activity of crude tissue preparations (3) we showed that in addition to the liver, the kidney also contains significant activity, one sixth (in rats) and half (in mice) as much per g as does the liver. The liver and kidney enzymes have similar kinetic properties.

Enzyme was not demonstrable in liver before the 21st day of gestation. At birth it was 20% of the adult value, and the latter was reached at 7 weeks of age. There was a sex difference, with male liver 20% higher than female liver. Kidney followed a similar development with age. The enzyme could not be induced prematurely in fetal livers. Control of the enzyme level in liver could be exerted by cortisol, but only in the neonatal period, raising the low level of enzyme to nearly the adult level. Increase in the enzyme concentration was paralled by increased rate of disposal of administered phenylalanine to tyrosine (4). In adult animals, inhibition of the enzyme by p-chlorophenylalanine slowed phenylalanine metabolism. Plasma levels of phenylalanine and tyrosine were found to be elevated in early life, in part related to simultaneous changes in the enzymes in liver acting on phenylalanine and tyrosine. These observations provide a firm foundation for further examination of the means of regulating gene expression of the phenylalanine enzyme, and for recognition of any enzyme variants.

Tryptophan Pyrrolase and Enzyme Degradation

A major effort exploited the properties of tryptophan pyrrolase to gain insight into the means and regulation of specific enzyme protein degradation. This was the first enzyme discovered to increase in tissues in

response to appropriate stimuli. Subsequent work under this contract and in other laboratories made this the best known instance of regulation of an enzyme concentration. It accumulated in rat liver with continued or increased synthesis of its protein, and then returned to its basal level by specific removal of this protein by unknown means. The advantages the enzyme offers for study of this important degradation reaction are its rapid turnover (half-life of about 2½ hours), inducibility to 10-times its basal level, and stabilization of its elevated level by the substrate. The latter is known to act by promoting conjugation of the protein with its heme prosthetic group, in which form the enzyme persists in the tissue as long as substrate is available. An hypothesis for control of the specific degradation of particular enzymes is immediately apparent. A non-specific degrading system might be able to act only upon non-functioning enzyme proteins, while combination with substrate, coenzymes, etc. could preserve any functioning proteins. This idea is consistent with general observations of "use hypertrophy" in biological systems, and has a chemical analogy in the resistance of native proteins to enzymic proteolysis.

Measurement of tryptophan pyrrolase as an antigen, titrating it by inhibition of its activity (or of an added indicator amount) with an antibody (9), freed the investigation from dependence upon catalytic activity as the sole quantitative indicator of the enzyme protein. It is of interest that 3 times more antibody was needed to react with the holoenzyme than with the apoenzyme. In livers of rats with induced high levels of the enzyme, quantitative measure of the enzyme protein was undertaken at intervals in the subsequent hours as the enzyme disappeared. The loss was followed in the intact rats, in perfused livers, in liver slices, and finally in cell-free

preparations (10). In all of these systems, the antigenic protein disappeared at similar rates that were proportional to its concentration, i.e., approximating the half-life in vivo. The protein was preserved in all the systems by its substrate.

^{14}C-labelled tryptophan pyrrolase prepared in rats was used to follow the enzyme that was lost in cell-free systems. It was converted to a catalytically-inactive, non-antigenic substance, that, however, retained its original molecular size (ca 100,000 molecular weight) and did not give rise to any detectable free amino acids or small peptides. The observations were interpreted as showing an obligatory "denaturation" of the protein, preparatory to its degradation by still unknown means of proteolysis.

Influence of Tumors on the Host Liver and the Chemical Differentiation of Rat Tissues

The stepwise appearance in successive clusters of the various enzymes that characterize adult liver has been described in previous reports. Each enzyme is evoked by a specific physiological stimulus that can be administered at an earlier age to evoke the enzyme prematurely. Such studies have given an unusually complete picture of the changing composition of liver at intervals during its maturation. Other tissues have also been examined as part of the study of each enzyme, so that a library of the enzyme patterns of each tissue has accumulated. Use was made of these patterns to reinvestigate the vexing problem of the effect of implanted tumors on the host. Increased mitotic activity and decreased catalase in the host liver are among the better documented changes, but none have been finally accepted as reproducible concomitants of tumor growth in the animal body.

Host Liver

The livers of tumor-bearing animals are known to exhibit a variety of biochemical abnormalities, including alterations in the concentration of several enzymes. Many of these changes could result from associated pathological conditions such as abnormal nutritional or hormonal states and tissue necrosis. However, our results indicate that the direction in which the overall enzymic composition of liver changes may be uniquely related to the presence of neoplastic growth in the organism (13). We measured the level of some 20 randomly selected enzymes in adult rat liver at 4 to 23 days after tumor transplantation, before the onset of major deterioration in the health of the animals. The results show that enzymes which increase in host liver upon tumor transplantation are among those that are relatively high in fast-growing hepatomas and in fetal liver. Those that decrease are those that are low or absent in hepatomas and fetal liver. Thus, by gain or partial loss of these enzyme activities, the quantitative pattern of enzymes in host liver diverges from normal adult liver toward that of immature liver and of the well-differentiated hepatomas.

To test whether the presence of a tumor in the organism might interfere with the normal process of enzymic differentiation, we implanted tumors into 10- to 14-day old rats and assayed, at intervals, some liver enzymes known to emerge around the 3rd postnatal week. The formation in liver of ornithine aminotransferase, glucokinase, glutamine synthetase, and malate-NADP dehydrogenase were inhibited. The effects of the tumor were qualitatively different from those of X-irradiation, starvation or adrenalectomy.

Fetal-type Isozymes in Neoplasms

Last year's progress report detailed the quantitative similarity of enzymes in tumors to those in fetal tissues, and the discovery of an apparent rule governing the qualitative composition of hepatomas: For enzymes occurring in different isozymic forms, the form present in hepatomas was also that form present in fetal liver. Some eight enzymes were known in which the isozymic variant in normal adult liver was different from the form in fetal and neoplastic liver. These instances were positive evidence that a significant part of the genome normally active during fetal life was reactivated in the hepatomas.

The same report described the evidence that generalized this rule in four tissue-tumor systems (liver, kidney, muscle and mammary gland) by chemical and electrophoretic analysis of the isozymes of lactate dehydrogenase, pyruvate kinase, and aldolase. National attention has focused on these results (2, 11) because they decrease the degree of empiricism in regards to tumors. With the earlier demonstration that the compositions of undifferentiated tumors from a variety of tissues were quantitatively similar, that several kinds of tumor had qualitative similarities, and that the composition of the tumors was like that of fetal tissues, both quantitatively and qualitatively, the nature of tumors began to lose its mystery. They can no longer be described as bizarre or lacking in chemical order. They have, in fact, a familiar kind of composition, one well-adapted to function, as they do, by growth with minimal control. The tumor compositions are not identical to those of fetal tissues, but the general similarity can provide a guide to the decisive differences.

From our surveys of the relative concentrations of enzymes in different tissues we identified two which were anomalous. Malate (decarboxylating) dehydrogenase was low or absent in fetal tissue but present in significant concentrations in tumors (12). Three isozymes of hexokinase (all with low Km's) can be separately electrophoretically, the slowest of which predominates in fetal tissues, but it is the fastest one which predominates in tumors (14). The latter isozyme also appears in liver of tumor-bearing rats, along with the other changes toward immaturity reported earlier (13). Further work is needed to appreciate the specific significance of these enzymes for tumor metabolism.

Search for still other isozymes (e.g. aspartate aminotransferase, (1)) was prompted by the above considerations, and what appears to be an isozyme of arginase was found in mammary gland. Attention was directed to this enzyme because it increased during lactation to one of the highest concentrations found in any non-hepatic tissue. Its appearance in the gland was not accompanied by any demonstrable amounts of two other enzymes of the urea cycle (5). A different function, possibly one leading to proline synthesis, must be ascribed to the mammary gland arginase. A different isozyme for a different function is a reasonable possibility. Electrophoretic separation of the liver and mammary gland arginases has since been achieved. Earlier experiments had shown that arginase, though low, is not absent from tumors, and identification of the type of enzyme in tumor and fetal tissues now becomes of interest.

Biochemical Definition of Tissue Enzymes

The systematic survey of tissues for an enzyme, after first perfecting

the assay for quantitative analysis, frequently reveals anomalies like that described for mammary gland arginase. These repeatedly suggest the occurrence of qualitatively new types of enzymes. Earlier measurements of glutamine synthetase, already reported, were subsequently checked by a second type of assay. Different reactions, both known to be catalyzed by purified glutamine synthetase, were used for the assays. They did not agree. Glutamyltransferase, measured by conversion of glutamine with ADP to the hydroxamate, was distributed more widely than glutamine synthetase, measured by conversion of glutamate with ATP to the hydroxamate. Simple fractionations showed that the two activities could be largely separated into the classical glutamine synthetase with some transferase activity, and a new source of very much more transferase activity, almost devoid of synthetase activity (15).

The same kind of resurvey of the tissue distribution of phosphorylase, using assays in opposite directions (to and from glycogen), gave completely concordant results. The absolute activity was 7 times greater in the direction of glycogen formation, but activities by both assays were strictly proportional. The results demonstrate that both assays are adequate quantitative measures of the enzyme, and that relative concentrations of an enzyme in several tissues can be reproducibly measured even though the absolute rates are not the same. This is the basis adopted for the comparison of results between different laboratories.

Enzyme Patterns in Normal and Neoplastic Tissues

All of the enzymes referred to above were surveyed as to their relative concentrations in a range of rat tissues, and a special effort was made to

add certain enzymes involved in nucleic acid synthesis to this study. The most complete study was of aspartate carbamoyltransferase, which catalyzes an early step in pyrimidine synthesis. Less complete results from earlier studies were corroborated in comparisons as relative concentrations, with a few exceptions.

The major extension of our knowledge of the distribution of aspartate carbamoyltransferase followed from recognition of a competing enzyme that destroyed the substrate in kidney, intestine and a few other tissues. The distribution, properties and location in the cells of this competing enzyme identified it as alkaline phosphatase, which apparently can hydrolyze carbamyl phosphate (7). When the assay was modified to avoid this artifact, a new species of the transferase was found, located in the particulate fraction of fetal and neoplastic tissues, but little or none in other tissues. The total activities, both soluble and particulate, correlated well with the growth rate of the normal tissues, and correlated significantly with the measured growth rates of the spectrum of tumors studied (8). These results support the expectation that more pyrimidine synthesis is required for cells growing more rapidly, and also show that the primary regulation of this enzyme in these tissues is not by more or less activation of an unchanged amount of enzyme, but by change in the concentration of the enzyme itself.

A preliminary integration of the many accumulated enzyme measurements which have been made in this laboratory was analyzed numerically according to the principles described in the book Enzyme Patterns of Fetal, Adult and Neoplastic Tissues (1972, Knox). The measurements were originally picked in order to test certain hypotheses (6). The first was self-evident, that undifferentiated fetal tissues chemically resemble one another more than do

the respective adult tissues. This was readily confirmed by correlation analyses using 21 tissue components. The same analysis could therefore test the analogous summary of Greenstein's work, that tumors resemble one another more than do the normal tissues from which the tumors arose. This was confirmed for undifferentiated tumors of liver and mammary gland, which were chemically very similar while the parent tissues were very different. The well-known diversity of tumors, which alone caused rejection of Greenstein's summary, was found among well-differentiated tumors. The composition common to tumors is apparently diluted in the differentiated tumors to a greater or lesser extent by the differing compositions of the tissues of origin. In this framework of tumors tending toward a common composition, the similarity of their composition to that of normal fetal tissues could be recognized. Extension of this type of analysis is expected to show other, more precise, relationships.

Other Activities

Lists of the lectures and other activities given by members of the laboratory during the period of this report are appended. This is an indication of the far-ranging instructional activity of the laboratory, as well as of the interest shown in its work. Less formal teaching exercises within the laboratory of postdoctoral fellows, graduate students and undergraduates are daily continuing events, pointed up by a weekly seminar given in turn by each resident in the laboratory about the current state of his work. This provides experience in presentations, but also an opportunity for guidance at formative stages of the work.

PUBLICATIONS SUPPORTED BY

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1. Herzfeld, A., and Knox, W. E. The distribution of aspartate aminotransferases in normal and neoplastic rat and mouse tissues. Enzyme 12, 699-703 (1971).

2. Knox, W. E., Farron, F., and Hsu, H. H. T. Fetal-type isoenzymes in rat tumors. Proc. Amer. Ass. Cancer Res. 13, 34 (1972). (Abstract 135)

3. McGee, M. M., Greengard, O., and Knox, W. E. The quantitative determination of phenylalanine hydroxylase in rat tissues; its developmental formation in liver. Biochem. J. 127, 669-674 (1972).

4. McGee, M. M., Greengard, O., and Knox, W. E. Liver phenylalanine hydroxylase activity in relation to blood concentrations of tyrosine and phenylalanine in the rat. Biochem. J. 127, 675-680 (1972).

5. Yip, M. C. M., and Knox, W. E. Function of arginase in lactating mammary gland. Biochem. J. 127, 893-899 (1972).

6. Knox, W. E. The protoplasmic patterns of tissues and tumors. Amer. Sci. 60, 480-488 (1972).

7. Herzfeld, A., and Knox, W. E. Carbamyl phosphate hydrolysis in rat tissues and tumors by an alkaline phosphatase. Cancer Res. 32, 1837-1841 (1972).

8. Herzfeld, A., and Knox, W. E. Aspartate transcarbamylase concentrations in relation to growth rates of fetal, adult, and neoplastic rat tissue. Cancer Res. 32, 1842-1847 (1972).

9. Li, J. B., and Knox, W. E. Titration of tryptophan oxygenase antigen by immunoinhibition. J. Biol. Chem. 247, 7546-7549 (1972).

10. Li, J. B., and Knox, W. E. Inactivation of tryptophan oxygenase in vivo and in vitro. J. Biol. Chem. 247, 7550-7555 (1972).

11. Farron, F., and Knox, W. E. Resurgence of fetal gene products in transplanted rat tumors. In: Embryonic and fetal antigens in cancer; proceedings of the second conference, February 14-16, 1972, Oak Ridge National Laboratory. Edited by N. G. Anderson, et al. Oak Ridge, Tenn., Oak Ridge National Laboratory, 1972, pp. 171-176.

12. Richards, T. C., and Knox, W. E. The distribution of malate NADP dehydrogenase in adult, fetal and neoplastic tissues of the rat. Enzyme 13, 320-323 (1972).

13. Herzfeld, A. and Greengard, O. The dedifferentiated pattern of enzymes in livers of tumor-bearing rats. Cancer Res. 32, 1826-1832 (1972).

14. Farron, F. The isoenzymes of hexokinase in normal anf neoplastic tissues of the rat. Enzyme 13, 233-237 (1972).

15. Huang, Y. Z., and Herzfeld, A. A glutamyl hydroxylamine transferase separable from glutamine synthetase in rat tissues. Federation Proc. 32, 472Abs (1973). (Abstract 1407)

LECTURES AND TALKS BY SCIENTISTS SUPPORTED BY

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W. E. Knox	5/4-5/6/72	Fetal-type isoenzymes in rat tumors, American Association for Cancer Research Annual Meeting, Boston, Mass.
W. E. Knox	6/7-6/11/72	Multidimensional Scaling, University of Pennsylvania, Philadelphia, Pa.
O. Greengard	7/18-7/20/72	"Regulation of Enzyme Amounts in Developing Liver" The Biochemical Society. Colloquium in Enzyme Induction, University of Surrey, Guildford, Surrey, England.
O. Greengard	9/26/72-2/2/73	Metabolic Regulations (course of lectures) Harvard Medical School, Department of Biological Chemistry. Biochem. 215.
W. E. Knox	10/21/72	American Cancer Society Council, Waldorf Astoria, Biochemical Detection of Cancer, New York, New York.
W. E. Knox	12/7-12/8/72	The Edwin H. Shaw Jr. Lectureship, "The Biochemical Nature of Cancer", and "Enzyme Regulation in Animals". The University of South Dakota, Vermillion, South Dakota.
W. E. Knox	12/12/72	"Enzyme Induction in Mammalian Systems". Harvard Medical School, Department of Biological Chemistry. Biochem. 716.0. Biochemistry Revisited.
O. Greengard	1/15-1/16/73	Lecture series on Biological Control Mechanisms: "Enzymic Differentiation in Mammalian Organs". Jefferson Medical College, Thomas Jefferson University, College of Graduate Studies, Philadelphia, Pa.
O. Greengard	3/14/-3/16/73	"The Effect of Hormones on Development of Fetal Enzymes". Symposium on Drugs and the Unborn Child at Cornell University Medical College, New York, New York.

W. E. Knox	4/13/73	Phenylketonuria. Harvard Medical School, Department of Biological Chemistry. Biochem. 709.
O. Greengard	4/12-4/14/73	"Enzymic Differentiation in Neonatal Rats". Conference on Problems and Priorities in Perinatal Pharmacology, Bethesda, Md.
W. E. Knox	4/18/73	Chairman of Isozyme Session at the F.A.S.E.B. Meeting, Atlantic City, New Jersey.