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

PROGRESS REPORT IX

W. EUGENE KNOX

NEW ENGLAND DEACONESS HOSPITAL
CANCER RESEARCH INSTITUTE
185 PILGRIM ROAD
BOSTON, MASSACHUSETTS 02215

1 MAY 1974 - 30 APRIL 1975

PREPARED FOR THE U. S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION
UNDER CONTRACT E(11-1)-3085

DISCLAIMER

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ABSTRACT

Investigations of the properties of variant forms of enzymes in rat tissues have continued. We purified two glutamyltransferases, one which remains associated with glutamine synthetase and the other which can be separated from it. A new assay method for glutaminase activity was established which facilitated further characterization of the 3 isozymes and their concentration in normal and neoplastic tissues. Continuation of our studies of arginase led to the demonstration of the role that the new variant of arginase plays in proline synthesis in mammary gland. An inhibitor of asparagine synthetase, which is absent from fetal liver and tumors, has been discovered in adult rat liver.

Peptidyl proline hydroxylase (an essential enzyme in collagen synthesis) has been identified as one of the most sensitive indicators of neoplastic growth. With a new method which affords more complete measurement of the particulate as well as the soluble activity we determined changes in the level of peptidyl proline hydroxylase in normal tissues during fetal and postnatal differentiation and in response to hormone treatment.

Our spectrum of experimental, transplantable rat tumors, has been extended to a series of salivary gland tumors and a radiation-induced lymphoma.

While continuing to study mechanisms of hepatic differentiation we began a systematic study of enzyme patterns and subcellular organelle differentiation in rat cerebrum. The seven enzymes so far studied throw some light on the changing composition of the cytosol, mitochondria and synaptosomes during early postnatal life.

Computer analyses of the accumulated data on tissue enzyme patterns show that only 3 or 4 well selected enzymes need to be measured in order to distinguish transplanted neoplasms from 24 normal tissues in the rat.

LEGAL NOTICE

Statement of Time or Effort of the Principal Investigator

During the current year of the project, the Principal Investigator, W. Eugene Knox, M. D., Member 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.

PROGRESS REPORT

Chemical Identification of Enzymic Variants

Recent studies in this laboratory have provided direct evidence for a clear distinction between the reaction forming L-glutamylhydroxamate from L-glutamate and ATP (GS activity) and that forming the same product from L-glutamine after arsenolysis (GT activity). In liver, 85% of the GS activity was released by water wheras 90% of the GT activity remained bound to particles unless extracted with deoxycholate or KCl. The question arose whether the GT activity, not separated from GS - now called GT(S) - represented a contamination of the water extract with the water-insoluble GT (GT(T)) - or whether the two GT activities were catalyzed by different proteins. The now accomplished purification of glutamine synthetase (GS) from rat liver demonstrates that a small portion of the GT activity remains associated with GS activity (GT(S)): as GS is purified from the water extract, the ratio between GT(S) and GS remains constant. The major portion of the GT activity (GT(T)) is left to be extracted by KCl from the pellet and, on further purification, appears to be independent of GS activity. Subtle differences in pH optimum, substrate requirement and reaction rates on addition of cofactors and amino acids in vitro and in responses to hormonal stimuli in vivo indicate that the glutamine transfer reaction may be catalyzed by two distinguishable proteins; only the minor component may be identical to GS (6).

In recent years, mammalian glutaminase has attracted wide interest because of the observation that isoenzymes with glutaminase activity are present in various rat tissues and tumors. A simple, reliable procedure is now described for the quantitative assay of glutaminase reaction by measuring product formation using an ammonia electrode (5). The ammonia electrode is a gas-detecting electrode, sensing the level of dissolved ammonia in aqueous solutions. Ammonia concentration can be read from calibration curves after converting ammonium ion to ammonia by adding sufficient base. Sample

color and turbidity do not affect measurements and samples need not be distilled. The concentration of the three glutaminase isoenzymes from rat tissues measured by this method are strictly comparable to those measured by other methods.

Asparagine Synthetase

Relatively low activity of asparagine synthetase has been reported in tumors sensitive to asparaginase treatment. Optimal conditions for quantitative assay of asparagine synthesis were now determined in fetal liver extract, which is a rich source of the enzyme. Demonstrable activity in liver fell 6 days after birth to 20% of the fetal value and decreased slowly thereafter to the low adult value. The asparagine synthetase of fetal liver extracts was significantly inhibited when combined with adult liver or tumor extracts. The inhibitor fractionated with ammonium sulfate is in close association with the asparagine synthetase (7). Therefore, demonstrable activities of asparagine synthetase in tissue extracts, measured in the presence of this inhibitor, do not necessarily parallel the concentrations of the enzyme present. The associations reported between measured activities of asparagine synthetase from tumors and the sensitivity of these tumors to asparaginase treatment suggest that slower asparagine synthesis makes for greater sensitivity to asparaginase treatment. This may mean that the inhibition observed in the assay *in vitro* also occurs *in vivo*, and that the inhibition described here represents a physiological mechanism of regulation.

Proline Synthesis

Our earlier studies suggested that the non-urea cycle isozyme of arginase that accumulates in mammary gland during lactation has a role in proline biosynthesis. The three necessary enzymes for this pathway, arginase, ornithine aminotransferase and $^{1}\Delta$-pyrroline-5-carboxylate reductase, were shown to accumulate in parallel and behave as a constant proportion group in mammary gland during lactation. The conversion was shown by incubating slices of lactating mammary gland with either labelled ornithine,

proline or glutamate and then separating the free amino acids with an amino acid analyzer. Labelled ornithine, but not labelled glutamate, after one hour incubation gave significant labelling of proline, while incubation with labelled proline or glutamate gave no counts in ornithine, thus indicating a net conversion of ornithine to proline (10). This conversion did not take place with slices of liver. Mammary gland slices obtained from rats at different times in lactation showed that, like the enzymes, the amount of conversion increased during lactation. These enzymatic and metabolic data show that the isozyme of arginase that occurs in most non-hepatic tissues could be involved in the biosynthesis of proline, at least in mammary gland.

Peptidyl Proline Hydroxylase (PPH)

The properties, subcellular distributions and tissue concentrations of this enzyme, essential for collagen synthesis, have been studied with a sensitive isotope assay (8). The results show that PPH is a very good indicator of neoplastic growth. In all transplanted tumors studied (renal, mammary, muscle and hepatic) the activities (per g tissue) were 4 to 10 times higher than in the cognate adult tissue. Even in well-differentiated, slow growing tumors the activity was considerably higher than in any normal, mature or immature tissue with the exception of the calvarium and lung of the 19-day-old fetus.

According to the only previously reported distribution of PPH among rat tissues (7 adult tissues were assayed as supernatants obtained by centrifugation at 15,000 x g, without Triton treatment), skin had higher activity than liver, kidney, and muscle. The present, more complete measurements of total activities raised liver significantly above the lower or similar values for skin, kidney, and muscle. The soluble activities of these 4 tissues were about the same. More importantly, only by assaying the whole homogenate after Triton treatment was it possible to show that brain contained very little soluble activity but as much total activity as did skin or kidney. High activities in the particulate fraction of brain homogenates, which includes the synaptosomes, is not unique for PPH. Thirty to fifty percent of the total activity

of several glycolytic enzymes in brain is also sedimentable by centrifugation (2). Since there is, unfortunately, no quantitative information available about the rates of collagen synthesis in different tissues, we cannot ascertain whether they correlate with the levels of PPH. The uniquely high activity in lung may be due to the synthesis of both basement membrane and interstitial collagen; however, intestine, which also produces both types of collagen, had the lowest PPH activity of all tissues tested. Lung was also rich (relative to kidney, muscle, brain, and heart) in glutamine hexosephosphate aminotransferase (Richards and Greengard. Biochim. Biophys. Acta, 304:842, 1973) necessary for glycoprotein synthesis. Both enzymes tend to be at high levels in growing tissues. The coprominence of PPH and glutamine hexosephosphate aminotransferase is most striking in the skull bone, where both enzymes are more concentrated than in any other fetal or neoplastic tissue. This probably reflects the rapid synthesis of both collagen and glycoproteins during osteogenesis.

Several examples indicate that, in different tissues of the developing organism, the upsurge or diminution of the same enzyme may occur at different times. Such asynchrony is to some extent also exhibited by PPH. While in brain it attained the low adult value 1 day before birth, in liver, kidney, and to a lesser extent in lung, there was a slow decrease throughout postnatal life. However, a sudden prenatal decrease in PPH activity is common to all 4 tissues examined and may be attributable simply to a decline in growth rate. There is evidence that the emergence at this time of some enzymes in liver is triggered by glucocorticoid secretion, which in fetal rats begins on the 16th day of gestation and becomes maximal on the 19th day. In rabbits, cortisol promotes pulmonary epithelial maturation (e.g., the capacity for surfactant synthesis) and at the same time inhibits cell proliferation. These results, together with the cortisol-induced loss of PPH activity from several fetal tissues observed in the late fetus, suggests that cortisol exerts 2 kinds of effects on development; while it enhances the differentiation of tissue-specific biochemical functions, it may also act as a nonselective inhibitor of growth.

The Graded Enzymic Immaturity of Neoplasms

Analyses of the characteristic constituents (e.g., the relative amounts of enzymic components) of rat tissues have been used to measure the degrees of chemical similarity among tumors. Undifferentiated, fast-growing tumors originating from the liver and from mammary gland have almost the same composition. Differentiated tumors from the same sources are more unlike in compositions, each kind tending to resemble the chemically very different parent tissues. A prototypic composition of tumors, whatever the source, appears to exist. When this is diluted by the various compositions of normal tissues, the products are tumors of lesser neoplastic character (more differentiated, slower growing, less autonomous tumors) (4).

The prototypic composition of tumors is very similar but not identical to that of many fetal tissues in both the quantitative patterns of enzymes and the qualitative identities of certain isozymes. It appears that a fraction of the cell genome acts in the same way in immature and tumorous tissues but differently in adult tissues. Measurement of the composition of selected tissues and tumors can distinguish a variety of tumors from a variety of normal tissues. Relatively small numbers of enzymes (4 or 5) are sufficient to make this important distinction between the 24 normal and tumorous tissues examined.

Initiation of New Lines of Tumors

Much work with salivary neoplasms has utilized primary tumors. Our purpose was to develop lines of transplantable salivary tumors with different growth rates and different degrees of anaplasia to serve subsequently as reproducible experimental models. Twenty NEDH/C strain adult male rats were implanted with 9,10-dimethyl-1,2-benzanthracene in the left submaxillary gland. Individual primary tumors began to appear between 11 and 32 weeks. Lines were initiated and maintained by transplanting pieces subcutaneously from each primary into 30-day-old NEDH/C rats. Histological characteristics were observed both in the primaries and the transplanted generations. These data were used to select permanent lines which would be most advantageous for study.

Detected. We obtained from Dr. Shields Warren a recently detected, radiation-induced, solid rat lymphoma. It grows well on serial transplantation but further studies are required to determine its volume growth, histological characteristics and stability during serial transplantation.

Cerebral Differentiation

Our studies of chemical and structural differentiation in rat tissues are now being extended to the cerebrum. The increasing concentration and changing subcellular distribution of seven enzymes have so far been followed as a function of age. During early postnatal development there is a preferential accumulation of glycolytic and some amino acid catabolizing enzymes in the particulate fraction, especially in synaptosomes (9). The maturation of mitochondria and synaptosomes involves the sequential addition of catalytic proteins to existing primary structures resulting in stepwise changes in functional potential. Such changes in the quality rather than in the quantity of subcellular compartments appear to play a crucial role in cerebral maturation during the third and fourth postnatal weeks.

Comparative studies were carried out between the fluorimetric (based on measuring the GABA formed) and the more sensitive isotopic assay (based on CO_2 release from labelled glutamate) for glutamate decarboxylase. We found that Triton not only increases the activity of this enzyme but also inhibits CO_2 release from glutamate via enzyme systems (present in all tissues) other than the glutamate decarboxylase itself. The use of this detergent circumvents the need for using a special apparatus to exclude oxygen from the isotypic assay system or for employing the more laborious, less sensitive fluorimetric method. We found that kidney and liver also contain some of the enzyme, in concentrations 15 and 10% of that in brain (2).

Enzymic Composition of Developing Chick Organs

In extending to other species the principles of chemical development found in the rat, the chick must be the first choice. The large amount of information about enzyme concentrations in chick organs scattered in the literature has not been adequately

analyzed and interpreted. In addition to carrying out some of the crucial missing measurements, we wrote a major review which classifies the enzymes in four chick organs (liver, brain, heart and kidney) on the basis of the developmental stage at which they emerge, scrutinizes the physiological relevance of the changing enzyme concentrations, and compares this material with analogous information we have about developing mammalian organs (3). It is hoped that the system designed for an objective assessment of the process of enzymic differentiation will benefit future studies of biochemical ontogeny in all higher animals.

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LECTURES AND TALKS BY SCIENTISTS SUPPORTED BY

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W. E. Knox	4/24/74	Anatomical Pathology, PalmerI: RE: Tumor Registry, New England Deaconess Hospital.
W. E. Knox	5/30/74	"The Lymphomas Updated", New England Deaconess Hospital.
W. E. Knox	8/18-23/74	"Fetal Enzyme and Isoenzymes". Symposium on Diagnostic Biochemistry and Cancer, American Association of Clinical Chemists. Las Vegas, Nevada.
O. Greengard	8/25-30/74	9th FEBS Meeting, Budepest, Hungary.
W. E. Knox	8/28-29/74	NIH Special Study Section, Sir Francis Hotel, San Francisco, California.
O. Greengard	9/2-7/74	"Enzymic Maturation in Rat Tissues". IV International Congress on Hormonal Steroids Symposium on Steroid Effects in Developing Tissues. Mexico City, Mexico.
W. E. Knox	9/11-14/74	Special Reviewer, NIH Molecular Biology Study Section.
W. E. Knox and O. Greengard	10/2-4/74	"Chemical Aspects of Tissue Differentiation" Harvard School of Dental Medicine. Oral Biology Course, 201.
O. Greengard	10/24-26/74	"Hormonal Regulation of Enzyme Synthesis in differentiating Mammalian Tissues". Symposium on Developmental Biochemistry-Inborn Errors of Metabolism. Eernewoude, The Netherlands.
O. Greengard	12/2-7/74	"Enzymic Differentiation During Hepatic Development". III International Symposium on Early Diabetes, Madeira, Portugal.
W. E. Knox	1/8 & 15/75	Harvard Medical School, Division of Medical Sciences. Tumor Biology. Biochemistry of Cancer I and II. Med. Sci. 204A.

W. E. Knox	2/3/75	"Experimental Basis for Fetal Markers in Neoplasms". Cancer Research Institute, New England Deaconess Hospital.
W. E. Knox	2/4/75	"Experimental Basis for Fetal Markers in Neoplasms". Boston Cancer Research Association.
O. Greengard	3/16-18/75	Temple University, Philadelphia, Pennsylvania.
C. C. Cappuccino	4/1/75	"The Development and Study of Transplantable Salivary Neoplasms". New York Meeting of the International Association for Dental Research and the American Association for Dental Research.