THE PARALLEL ACCUMULATION AND DISTRIBUTION OF TWO PURINE-OXIDIZING ENZYMES DURING FROG DEVELOPMENT

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> "As for myself, I like only basic problems, and could characterize my own research by telling you that, when I settled in Woods Hole and took up fishing, I always used an enormous hook. I was convinced that I could catch nothing anyway, and I thought it much more exciting not to catch a big fish than not to catch a small one."
>
> --Albert Szent-Györgyi (113)

However little has been caught, the chase has been most rewarding, and I am heartily grateful to all those who helped. In particular I wish to thank Professor Herschel K. Mitchell for his inspiration and counsel, and for his kind and tolerant guidance throughout this work. I am also indebted to Professors Ernst Hadorn and Pei-Sei Chen for their hospitality in the Zoological Institute of the University of Zürich where this work was begun; to Doctors Robert L. Metzenberg and Alexander Miller, and Mr. Paul C. Denny, for practical advice and stimulating discussion during various phases of the research; to Professor Norman H. Horowitz for assistance in the preparation of this thesis; and to Mr. Roger W. Hendrix, my undergraduate apprentice during the summer of 1962.

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ABSTRACT

The patterns of two specialized and functionally related enzymes were investigated in the developing frog (Rana temporaria and Rana catesbeiana). Xanthine dehydrogenase (E.C. 1.2.1.) and uricase (E.C. 1.7.3.2.) were found to remain apparently constant during the first ten days of normal development, both rising rapidly thereafter, beginning at the same larval stage. Comparison with enzymes of similar developmental pattern suggests this phase-specific increase is in preparation for the onset of digestive activity.

The same parallelism between uricase and xanthine dehydrogenase was observed when determinations were extended to the organ level. Each enzyme was found in quantity in liver and kidney, and in trace amounts, all of about the same magnitude, in several other organs tested.

By detecting radioactive tracer amounts of product, very low levels of xanthine dehydrogenase were measured in embryonic stages, probably corresponding to the order of small numbers of molecules per cell.

Evidence was gathered to suggest that embryonic uricase accumulation is neither induced by its substrate nor involved with the subcellular particles in which the enzyme is found.

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INTRODUCTION : DEVELOPMENTAL ENZYMOLOGY

A. The Roles of Enzymes in the Study of Differentiation

1. Enzymes as Agents of Differentiation

Cytodifferentiation. The view of some early developmental enzymologists, that studies of enzyme concentrations and mechanisms would suffice to clarify the mechanisms of embryonic differentiation, was well summarized by Shen in 1955: "Specific enzymes are most probably involved in the synthesis and transformation of specific proteins. From this point of view the study of developmental enzymology contributes directly to a causal analysis of proteinogenesis as a chemical basis of morphogenesis." (99). Shen went on to caution, however, that knowledge of protein synthesis was not yet sufficient to support any speculation on the role of enzymes as regulators of ontogenetic changes. Later reviews in the field have generally echoed this caution (cf. (100)), or minimized the role of enzymes as direct agents of the differentiative process (46, 120, 73). Of the former writers, for instance, Moog has suggested that intracellular structural relationships will have to be taken into account before a causal scheme for cellular differentiation can be extrapolated from simple descriptive enzymology (74, 110).

The recent identification of many of the protein biosynthetic enzymes (amino-acid activating and transfer enzymes, RNA polymerase) promises new insights into the problem. If the pattern of proteins present in a tissue is accepted as the end product of differentiation, then the protein-synthesizing and -degrading enzymes are indeed the agents of the process. The possible contributions of mitochondria and energy-producing enzymes to specificity in development has also been mentioned (120, 80). But to what extent any of these enzymes are specific, and able in their interactions to regulate the rate of production or destruction of one particular protein over another, remains a question. Its experimental resolution now seems in sight.

Holodifferentiation. Grobstein (42) has pointed out the semantic problems with the word differentiation and has suggested the terms "cytodifferentiation" for the intracellular process in which functional diversity generally decreases, and "holodifferentiation" for the process at the level of the organism, which achieves diversification.

That enzymes must be agents of holodifferentiative processes may be obvious, but one well-understood example is worth citing. The resorption of the anuran tail in metamorphosis is accomplished by manifold-increased activities of a number of hydrolytic enzymes, as has been reviewed in detail recently (5).

2. Enzymes as Indices of Differentiation

Approach. The large part of the literature of developmental enzymology has concerned the enzyme as a thing in itself, a tangible criterion of the progress of differentiation. The assay of enzyme (or equally well, antigen) concentration offered an escape to a quantitative basis, from the qualitative observations to which classical embryology was largely limited.

The unit experiment in this field comprises a series of assays of a single enzymatic activity, in individuals of one stock, at frequent intervals during the time of early development, each determination referred either to weight of protein or to the single animal. The attack is purely descriptive in concept, as several reviewers have emphasized (121, 58, 99). There is implicit in such work, however, the thought that biochemical description of ontogeny will not merely parallel morphological description of ontogeny. Rather, in Shen's words, "...in chemical embryology as in classical embryology, accurate and adequate descriptions of a phenomenon are the indispensable groundwork without which experimental and causal analyses of morphogenetic processes are neither possible nor valid" (99).

As reports on various enzymes in ontogeny have accumulated, the information from unit experiments has been compared, and interesting generalizations have emerged which will be treated briefly below.

Accumulation Patterns of General Enzymes. The earliest developmental enzymatic studies were refinements of the many developmental studies of respiratory rate (reviewed in (7)) and hence concerned respiratory enzymes. Such enzymatic activities were generally found to increase in a continuous fashion, reflecting only the general uniform increase in functional capacities concomitant with transformation of the vitelline storage material into "active protoplasm" (7, 58). Weiss as late as 1953 registered the criticism that embryologists had favored for developmental study these ubiquitous enzymes engaged in cellular maintenance metabolism (121).

Specialized Enzymes and Phase Specificity. In 1943 Sawyer published an investigation (95) of the ontogeny in the whole amphibian embryo of cholinesterase, a specialized enzyme concerned with myoneural function. Cholinesterase activity was reported to increase discontinuously, remaining negligible until a time which could be correlated with the beginning of complex muscular functions. The growth of interest in the temporal patterns of specialized enzymes' increase is shown in the extensive review by Urbani (117).

Such observations are more useful when related to an enzyme's functional significance. In 1953 for instance Løvtrup presented his graphs of the ontogeny on a time axis of 11 separate enzymes (57), and divided these into five classes according to the phase when each began to increase. The five phases were then

correlated with contemporaneous morphogenetic events, and the enzymes were found in general to perform functions which could be associated with newly differentiated structures.

Wallace's recent investigation of the ontogeny of 12 enzymes mainly of a respiratory nature (118), may serve to modify the notion of general and specific enzymes in two ways. First, Wallace's early assay points considerably precede those of earlier workers and show that enzymes which would be considered general,increase from an early time but not from fertilization. Secondly, other enzymes which are certainly involved in cellular maintenance metabolism are shown to arise quite late in ontogeny. Hence the line demarking general from specialized enzymes on temporal criteria is not a sharp one.

Spatial Specificity. Another parameter of specialization was early recognized: an enzyme which arose only at the time when it was needed was likely too to be restricted to the place where it was needed. Thus Sawyer, having correlated the time of increase of cholinesterase in the whole animal with the beginning of controlled motor function (95), proceeded to demonstrate that the enzyme was detectable only in neural and muscular tissues of the embryo (96). The principle that spatial and temporal specificity tend to go hand in hand appears to have general significance, and has been explored

by several writers (72, 99). Moog has reviewed a number of examples in amphibian, avian and mammalian systems (74).

Both Moog and Shen have also noted that as differentiation proceeds and enzymes concentrate in different tissues, measurement of whole-embryo activities becomes less meaningful (72, 99). The limited use an enzyme may have as a criterion of holodifferentiation (as, e.g., of when yolk is transformed into cytoplasm (7)) gradually wanes as the enzyme becomes more useful as an index of cytodifferentiation. As the embryo increases in complexity, the level of interest changes. It becomes necessary to examine it on more levels and to correlate findings trans-level, a phenomenon which Grobstein has called fundamental to embryological inquiry (42).

Another corollary to the notion that enzymes appear when and where functionally pertinent is that enzymatic activities may decrease when their functions are superceded. After one case reported in 1935 (77), such cases were rare and the possibility of the loss of an enzyme, once accumulated, was neglected until quite recently (55, 72, 94, 63).

In the spatial realm, the distinction between general and specific enzymes is more useful. Greenstein, for instance (40), has tabulated the distributions of many enzymes among 16 mammalian organs. The enzymes of his sample fall readily into two classes,

those evenly distributed, and those considerably concentrated in from two to four of the organs assayed.

Frequently an enzyme which would fit the category described as specialized, duplicates the function of a general enzyme, rising more precipitously and in a more restricted area (see below p. 26). Moog has drawn an intriguing analogy between the ubiquitous acid phosphatase and the more restricted alkaline phosphatase of her studies (74), and the constitutive and adaptive enzymes of microbiological control systems.

Spatial and Temporal Specificity. A decade ago Moog made two predictions relating embryonic changes of functionally specialized enzymes to both parameters, space and time (72). She predicted first that the same enzyme would be found to exhibit different temporal patterns of increase in different organs, and secondly, that within a single organ several functionally associated enzymes would be expected to have similar patterns. These points were intended simply to elaborate the principle that enzymatic competency accompanies functional differentiation, and to strengthen it by bringing more critical evidence to bear. The principles are quite significant from the standpoint of mechanism, however, so that it is regrettable that experimental designs of sufficient complexity to apply to them have been scarce in recent work. The evidence can be summarized quite briefly.

The first principle, that an enzyme shows varying accumulation patterns in different tissues, resulted from Moog's histochemical studies on alkaline phosphatase in a wide variety of developing chick tissues (70). It has been borne out by a quantitative study contrasting ATPase development in chick muscle, brain, and liver (71), and by a similar investigation of carbonic anhydrase in chick retina, stomach, and kidney (17). Solomon's determinations of three dehydrogenases in four developing chick organs (103, 104) also show quite divergent patterns.

Moog has implied the possible general significance of the chick alkaline phosphatase pattern, where a moderate enzyme activity is found in undifferentiated tissues, but upon differentiation each tissue, in accordance with its adult pattern, either gains considerable activity or loses the enzyme altogether. This scheme still rests on histochemical determinations, and no other developing enzyme has been described in enough detail to confirm it.

The second principle, that functionally associated enzymes in the same organ develop in similar patterns, shifts the burden of proof to the experimenter. Moog, however, was able to cite evidence for the parallel accumulations of acid phosphatase (70), peptidase (28), and glutamotransferase (92) in chick liver at the fifth to eighth days of incubation. Solomon's data show that malic and lactic (104) and glutamic (103) dehydrogenases rise in the chick liver in similar

curves, but they diverge from the first group cited, in starting later and climbing more gradually. Flexner has shown that ATPase, several respiratory enzymes, and aldolase all rise suddenly in the guinea pig cerebral cortex at the critical period of neuronal differentiation (32). The most satisfying study of enzymes functionally associated at the biochemical level is Brown and Cohen's case of the five enzymes which compose the Krebs-Henseleit urea cycle. They have shown (10) that activities of all five enzymes increase in tandem in the liver, at the stage in frog metamorphosis when ureotelism must supplant ammoniotelism.

3. Enzymogenesis as a Model of Ontogenesis

"In a crude way we can correlate the patterns of enzyme accumulation that have been delineated with what we know of the activities or needs of the tissue under study. The explanations thus arrived at are,however, of a somewhat teleological flavor and of limited value in probing the underlying mechanisms." (Moog (73))

To a biochemist, the most promising approach to the underlying mechanisms of differentiation may consist in a direct inquiry into the ontogenetic changes of the factors controlling the synthesis of one (and eventually many) model proteins. That these factors may include synthesizing enzymes was a focus of attention in section 1 above. Wherever the controls lie, enzymes may be useful in

discovering them, serving as model protein products, for reasons of convenience and sensitivity of assay. Whereas the stress was placed in section 2 on the enzyme as a finished product to be inventoried, here attention is directed to the processes preceding and resulting in the enzyme's appearance, and to the possibilities for their experimental manipulation.

Presumably this approach will be vigorously pursued in the near future, but most of the elements of the current understanding of protein synthesis are of such recent discovery that they have not yet been investigated in embryonic materials. Hence the only results which will be mentioned are those of attempts to implicate substrates as inducers of specific enzyme syntheses, which date back to Spiegelman's suggestion that microbial control mechanisms could be generalized to explain embryonic systems (108). Apparently successful inductions have been achieved in a few embryonic tissues (3, 38), but in other cases dependable negative results have been obtained where inducibility was expected (31, 18, 79, 51). Hence the significance and generality of the phenomenon remain in doubt. Nemeth's experiments perhaps foreshadow a general solution to the problem. In guinea pig and rabbit, tryptophan peroxidase is present in adult liver, its level partly controlled by substrate induction (53). In the fetal liver, however, the enzyme is at a very low

level, and cannot be induced. Nemeth has shown that immediately after birth the abrupt rise from embryonic to adult tryptophan peroxidase levels occurs during a 24 hr period, and only with the accumulation of the enzyme comes the capacity for further induction by substrate (78). In this case, then, substrate induction can be recognized as a phenomenon of physiological, but not embryological, significance.

B. Methodological Problems with Enzyme Assays

1. Misestimation of *in vivo* Activities

Embryonic enzyme determinations are conducted with the varying goals detailed above, and the hazards in extrapolating from *in vitro* assays vary. If the enzyme is of interest as an agent of development, then the assay should ideally reveal the activity of the enzyme *in vivo* and indeed, *in situ*. To approximate *in vitro* the microenvironment in which the enzyme operates within the cell is at present impossible. The process of homogenization destroys structural relations which may sterically aid the enzyme in its catalytic function (101, 45 for references) or alternatively may impede substrate access (109, 23); so that assaying a homogenate may either under- or overestimate the physiological efficacy of an enzyme. That

in vitro assays are routinely conducted with optimal substrate, activator, hydrogen ion and salt concentrations tends, of course, to overestimate the activity. Brown and Cohen, seasoned enzymologists, have estimated that to a first approximation observed rates should differ by less than an order of magnitude from effective physiological rates (10).

George Weber has given a very thorough discussion of the pitfalls inherent in such extrapolations, and has stressed the value of supplementary in vivo and tissue slice assays (119).

2. Derivation of Enzyme Concentrations

If on the other hand the enzyme is studied as a product of differentiation, then the goal, to determine the concentration of a specific protein molecule, is elusive but within the limits of present technique. Once the enzyme is soluble, its potential activity, as measured under optimal conditions, should bear a constant relation to its concentration, so that activities (with zero-order kinetics) are as useful for comparative purposes as are exact concentrations. The two quantities can be related, through the molecular activity (turnover number), as soon as the enzyme is obtained in pure form, but this has not actually been done in any developmental study to date.

Bound Forms. Enzymes' solubilities can change during ontogeny, as Spiegel has shown (107), and in particular, recent

investigations of several enzymes have shown that they shift their location, at the subcellular organelle level (105, 120). If the enzyme preparation is assayed in a crude enough state that both bound and free forms of enzyme will catalyze (with different efficiencies), comparisons at different points in ontogeny will be misleading, to the extent that a change in localization may be misinterpreted as a change in concentration.

Activation from Precursors. Enzyme activation from an inactive precursor can also invalidate the correspondence between enzyme activity and concentration, the changes in a time curve reflecting the relatively nonspecific activation step rather than specific protein biosynthesis. This effect is well known in the proteinases, so that most developmental enzymologists have felt obligated to consider it, if not too seriously (74, 58, 55). The extreme view, that all enzymes needed before hatching are stored in the egg as inactive preenzymes, is reduced to absurdity by various reports of early effects of paternal genes, particularly Ranzi's demonstration of paternal antigens in cleavage stages (86).

If enzymatic proteins are not synthesized in the embryo, Løvtrup has argued (58), they will have to be pictured as a quite separate class from structural proteins, whose increase is visible. Thus the general tendency to minimize the differences between

structural and catalytic proteins (gaining impetus from Englehardt's demonstration of myosin's ATPase activity (30)) has not favored attempts to read developmental significance into preenzyme activation.

Nonetheless for any given enzyme, the suspicion that an activation effect obscures assay results can only be ruled out completely by experimentation. This has been accomplished rigorously in only one case, where Metzenberg et al. have shown that increased carbamyl phosphatase activity is paralleled both by increase in radioleucine incorporated into the enzyme molecule, and by increase in antigen giving the precipitin reaction with anti-enzyme antiserum (68).

Multiple Molecular Forms. Finally, in translating enzymatic activities into concentrations, or comparing activities, it is assumed that a single species of protein is synthesized throughout ontogeny. Following the pioneer recognition of their importance by Markert and Møller (65), multiple molecular forms of enzymes within the same tissue have been found in many instances (127), but with particular relevance here, embryonic forms have been found which differ from adult forms (76, 12, 56) in substrate specificity. If such a multiplicity of forms goes unrecognized, the assays will obviously lose accuracy. Precautions like determining the Michaelis-Menten constant at several points in ontogeny (e.g. (120))are useful insurance measures.

3. Limits of Sensitivity

The problem of sensitivity in the lower ranges of normal assay techniques has not attracted much interest among developmental enzymologists. Enzymatic activities below the sensitivity of the method have generally been labelled zero, and the sensitivity limit seldom recorded. Whether values below these "zeroes" might correspond to significant intracellular activity is usually an open question, as was discussed above, but they would be of considerable importance to the student of control mechanisms.

This imprecise reporting of low activities has led to unwarranted conclusions. Markert, for instance (as reported by Rudnick (64)), has asserted that "the acquisition of distinctive metabolic patterns by cells during the course of differentiation involves qualitative losses in synthetic activity and qualitative gains, as well as shifts in relative synthetic activity." More cautious authors have pointed out that the inherent limitations of classical enzymatic assays do not permit a negative result to be interpreted as total absence of the enzyme. Thus whether the synthesis of any qualitatively new proteins occurs in development has never been proved experimentally, and has on occasion been brought into doubt (46, 58). (On theoretic grounds, the case of the F_1 from a female homozygous recessive by a male homozygous dominant should be instructive here.)

At least for the newly fertilized egg, Moog has suggested that

techniques may never be adequate to reveal a "small number of enzyme molecules carefully shielded within the volume of an ovum." (73)

C. Basis for the Present Work

The time period in embryogeny before the establishment of a specialized enzyme appears to offer a particularly attractive working place, affording the student of the dynamic gene an experimental situation formally analogous to the recessive mutant in the analysis of the static gene.

It was therefore decided to seek out a phase-specific enzyme and to describe its distribution in space and time, by methods of adequate sensitivity to pave the way for eventual inquiry into the mechanisms directing its changing concentration.

The frog embryo, available in large quantities, conveniently manipulated, and well investigated on a morphological level, was chosen as the subject of these investigations.

PART I : SPECTROPHOTOMETRIC STUDIES ON URICASE ACTIVITY IN DEVELOPING RANA TEMPORARIA

A. Introduction

1. Choice of Enzyme

On the basis of preliminary investigations, uricase ("Urate: O_2 oxidoreductase," number 1.7.3.2 in the list of the Commission on Enzymes (49)) was chosen over a number of other enzymes tested, for its late appearance of activity and its ease of assay.

When this work was nearing completion, it was learned that a preliminary exploration of the ontogeny of uricase had been recorded in the literature of thirty years ago (113a).

Uricase catalyzes the oxidation of urate to allantoin and CO_2 in the following overall reaction:

$$Urate^{2-} + 2H^{+} + O_2 \longrightarrow Allantoin + CO_2 + H_2O_2$$

Discussion of the complex series of steps which are theorized to make up the overall reaction (61) will be deferred to a later section.

2. Physiological Significance

The position of uricase in the purine catabolizing pathway is shown in figures 1 and 2. Its biological importance to frogs is not

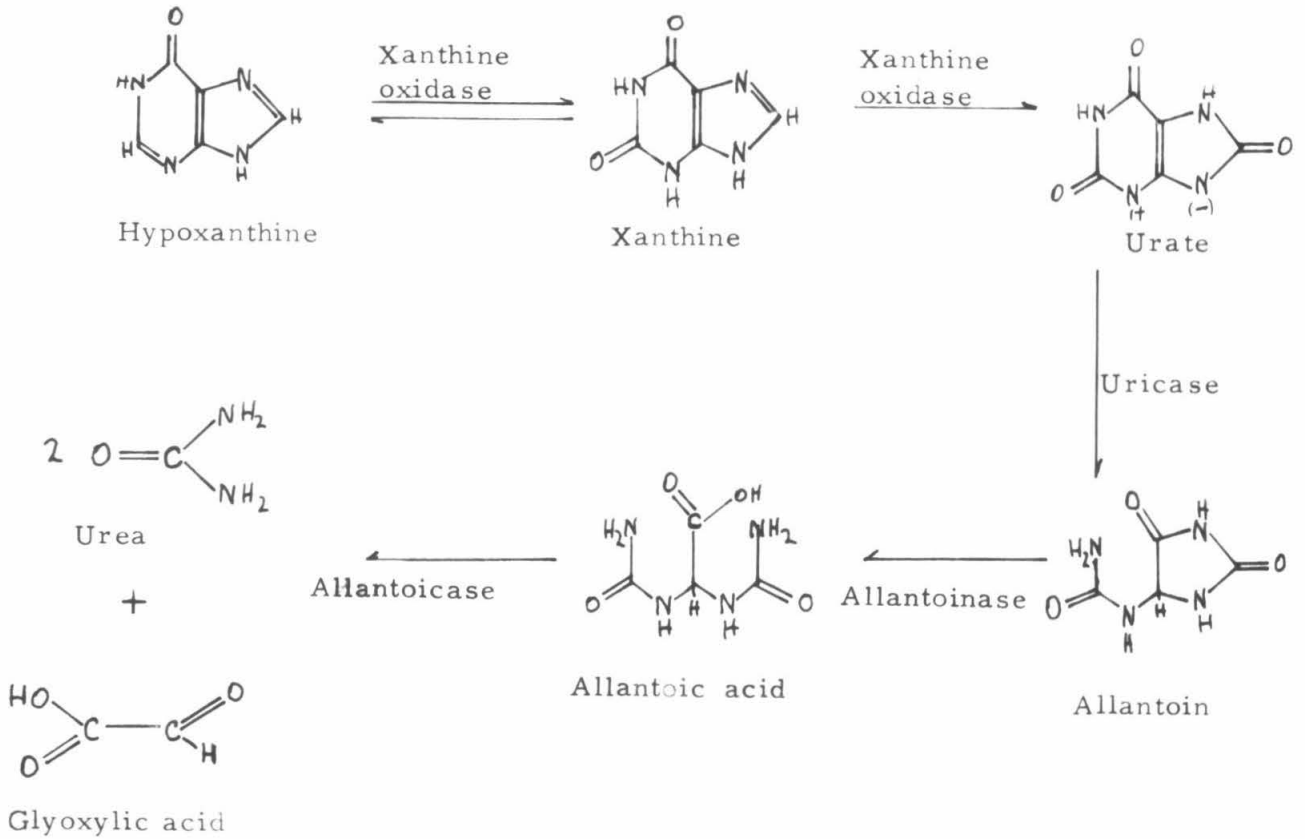


Fig. 1. Terminal purine catabolism in amphibians, after Brunel (11).

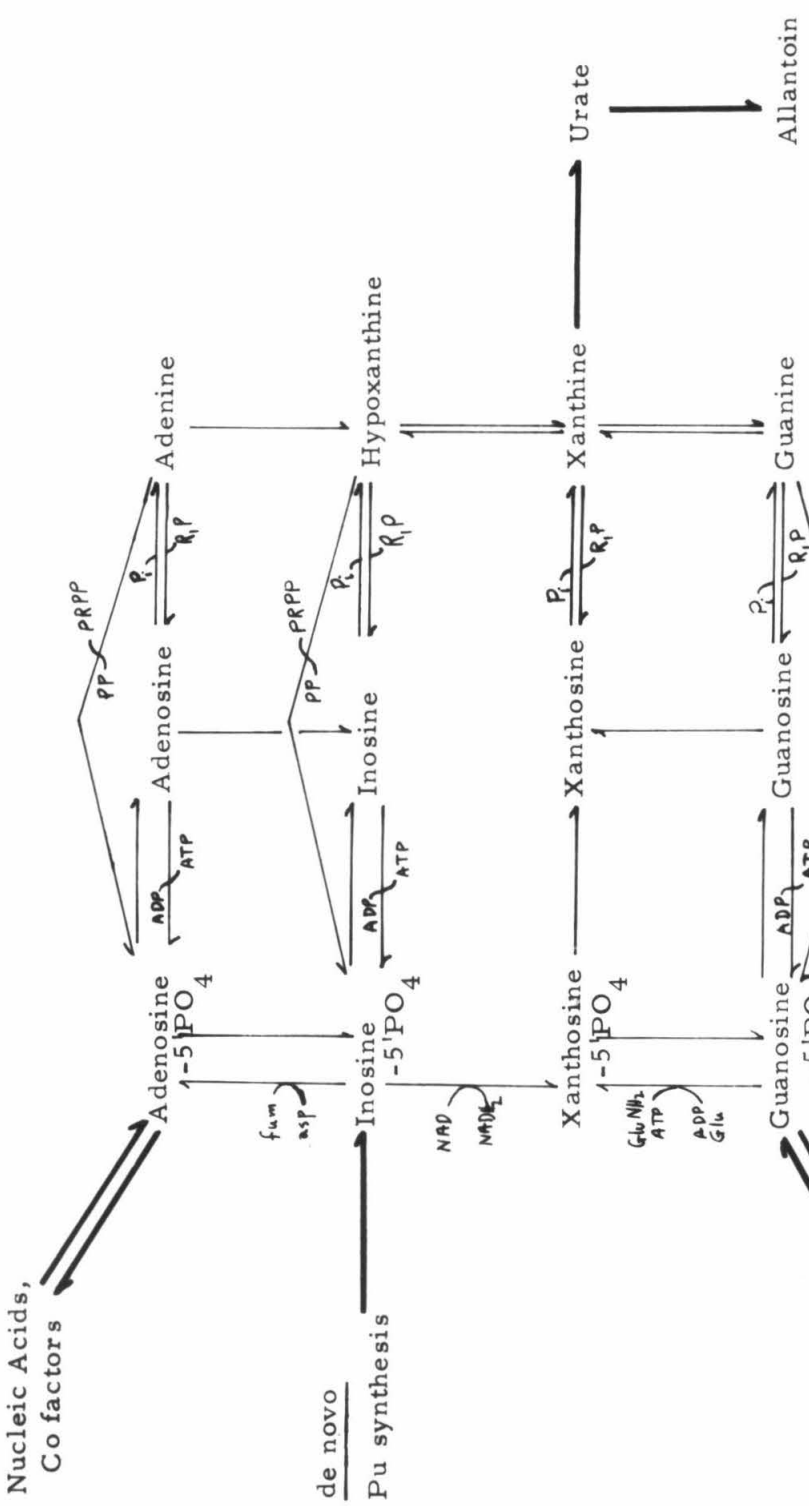


Fig. 2. Purine catabolic pathways which have been found in animal systems. General significance is not claimed for all reactions. After data of Schulman (98).

a priori evident. Neither urate, nor urea formed from urate (through allantoin, by uricase) is a major excretory product. Brown and Cohen have shown (10) that the enzymes of the Krebs-Henseleit urea cycle are responsible for urea formation, in minor amounts in early ammoniotelic stages, and in large quantities after metamorphosis, when the animal is ureotelic. Functional uricotelism, as it has evolved in the organisms with cleidoic eggs, where purine synthesis and oxidation serve as the major nitrogenous excretion pathway, is never operative in frogs.

Yet it would seem that once the small amount of purine waste had reached the oxidation level of urate, it should be an acceptable excretion product. The value of converting urate to urea, requiring three enzymes (uricase, allantoinase and allantoicase) and freeing only CO_2 and glyoxylic acid in the process, is not obvious. No useful energy capture has been postulated for the process.

In fact, adult frogs, despite their uricase content, are normally able to excrete a small amount of urate (0.2 per cent of total N excreted by Rana esculenta (85) and a similarly small proportion by R. catesbeiana (106)), by both glomerular filtration and tubular secretion (59). When exogenous urate is injected, the more than half which escapes uricolysis is excreted (85).

B. Methods and Materials

1. Biological Stocks

Rana temporaria* eggs were collected from ponds in the neighborhood of Wintherthur, Switzerland, in the early spring of 1960; later in the season parents in amplexus were shipped from Andermatt and eggs were laid in the laboratory. The stocks were raised at 18°C and the larvae fed on Brennesselpulver, a nettle powder available from the apothecary.

Early metamorphosis was induced in one clutch by adding 0.02 µg/ml of Hoffman-LaRoche thyroxin to the culture water.

2. Homogenization and Photometric Assay

The most convenient assay of uricase employs differential spectrophotometry according to the methods described by Kalckar (50). The reaction is followed by measuring absorbance (optical density) at 292.5 mµ, where urate has its λ_{max} and allantoin is transparent, against an enzyme blank without substrate. At 9.2, pH was optimal for enzymatic activity (the peak is broad), and 9.2 is superior to lower pH at least in retaining activity during the

*Kopsch has divided the species temporaria into two very similar species fusca and arvalis, and has concerned his excellent graphic description with Rana fusca development (54). From his descriptions it is probable that the species used in this work was fusca, but failing positive identification, temporaria is written, under which name the frogs were supplied, and which designation is still widely used in the literature. Kopsch's normal table (54) is used for all stage designations in this paper unless another source is cited.

homogenization process; hence Na glycinate at pH 9.2 was used for both homogenization and assay.

The concentrated homogenates used in order to detect minimal activities introduced a high blank absorbance into the assay, as well as appreciable buffer capacity at a lower pH. To combat the pH effect the assay buffer was made up to 0.8 M, when experiments showed the activity to be virtually independent of ionic strength within wide limits. Subsequently the crude extracts were subjected to dialysis against 0.1 M Na glycinate (pH 9.2), 4°C, without loss of activity, and this step obviated both problems.

A substrate concentration of 8×10^{-5} M is nearing enzyme saturation, while still within the linear response range of the spectrophotometers used, and was employed in all determinations.

Protocol. 1. Homogenize 5 to 20 embryos in an approximately equal volume of 0.8 M Na glycinate (pH 9.2), in a ground glass cone grinder in ice, for 2 min, at 1000 rpm, top speed on the Precision Scientific Co. Vari-Speed Stirrer, or its equivalent.

2. Centrifuge the homogenate 2 min X 15 K g, either full speed on a microcentrifuge obtainable from Ole Dich, 18 Holmevej, Brøndby strand, Denmark; or at 19 K rpm with head 295 and the multi-speed attachment, on the International Refrigerated Centrifuge, Model PR 2.

3. Twice dialyze the supt against at least 50 volumes of 0.1 M Na glycinate (pH 9.2), over a period of 6 hr to 2 dy at 4°C.

4. Add 0.060 ml of the retentate to

0.010 ml of 12.5mM Na H urate and

1.830 ml of 0.1M Na glycinate (pH 9.2)

5. Read absorbance at 292.5 mμ in the Beckman DU Spectrophotometer in a 1 cm path-length cuvette, against a blank cuvette of

0.010 ml of retentate and

1.890 ml of buffer.

Later the assay was scaled down, and run in a total volume of 0.335 ml in a microcuvette in the Cary Model 11 Recording Spectrophotometer.

Linearity. Absorbance loss was linear with time well past the 30 min the reaction was routinely followed. The slopes were proportional to enzyme concentration up to a velocity of 0.06 O.D./min.

Calculations. The unit (U) of uricase activity was defined, in keeping with the report of the Commission on Enzymes of the International Union of Biochemistry (49), as that amount which will catalyze the transformation of 1 μmole of urate per minute at 30°C and pH 9.2, extrapolated to infinite substrate concentration.

The factor for extrapolation to infinite substrate, $\frac{V}{v}$, is derived from the Michaelis-Menten relation

$$v = \frac{V[s]}{K_M + [s]} \qquad \text{by rearrangement, } \frac{V}{v} = 1 + \frac{K_M}{[s]} .$$

The K_M under the conditions of the assay was determined as 4.3×10^{-5} M, hence the correction applied was

$$1 + \frac{4.3 \times 10^{-5} \text{ M}}{8.0 \times 10^{-5} \text{ M}} = 1.58 .$$

The millimolar extinction coefficient was taken as 11.6 (14). Hence for a typical calculation,

$$1 \text{ U} = \frac{1 \ \mu\text{mole}}{\text{min}} \times \frac{11.6 \text{ O.D./mM}}{1.9 \text{ ml} \times 1.58} = 3.87 \ \frac{\text{O.D.}}{\text{min}} .$$

C. Results

1. Ontogeny of Uricase

Normal. Curve a of figure 3 shows the development with time of uricase activity in whole animals of a typical sibship. The striking fact, that activity is undetectable until it begins to rise steeply at about the tenth day, in stage 18, was confirmed in several clutches. Large numbers at stage 18 were taken from one clutch, to ascertain that activity was nil in larvae 12.5-13.0 mm long, but measurable in larvae 13.0-13.5 mm and longer. It is at just this turning point that the intestine first appears green with ingested Brennesselpulver.

Genetically homogeneous amphibian stocks are not yet available. Variation within a sibship increases as time progresses, especially after the start of feeding, and later points, too, generally represent fewer animals. Thus it was not surprising that the slope of the ascending curve was not quite constant from clutch to clutch. The data plotted in figure 3 are representative, not average.

Unfed. From the scatter in later points, it was suspected that nutritional differences might be a controlling factor. Coupled with the timing of the first appearance of the enzyme, this suggested that food, perhaps something specific in Brennesselpulver (which contains purine derivatives of pharmacological interest), might be inducing

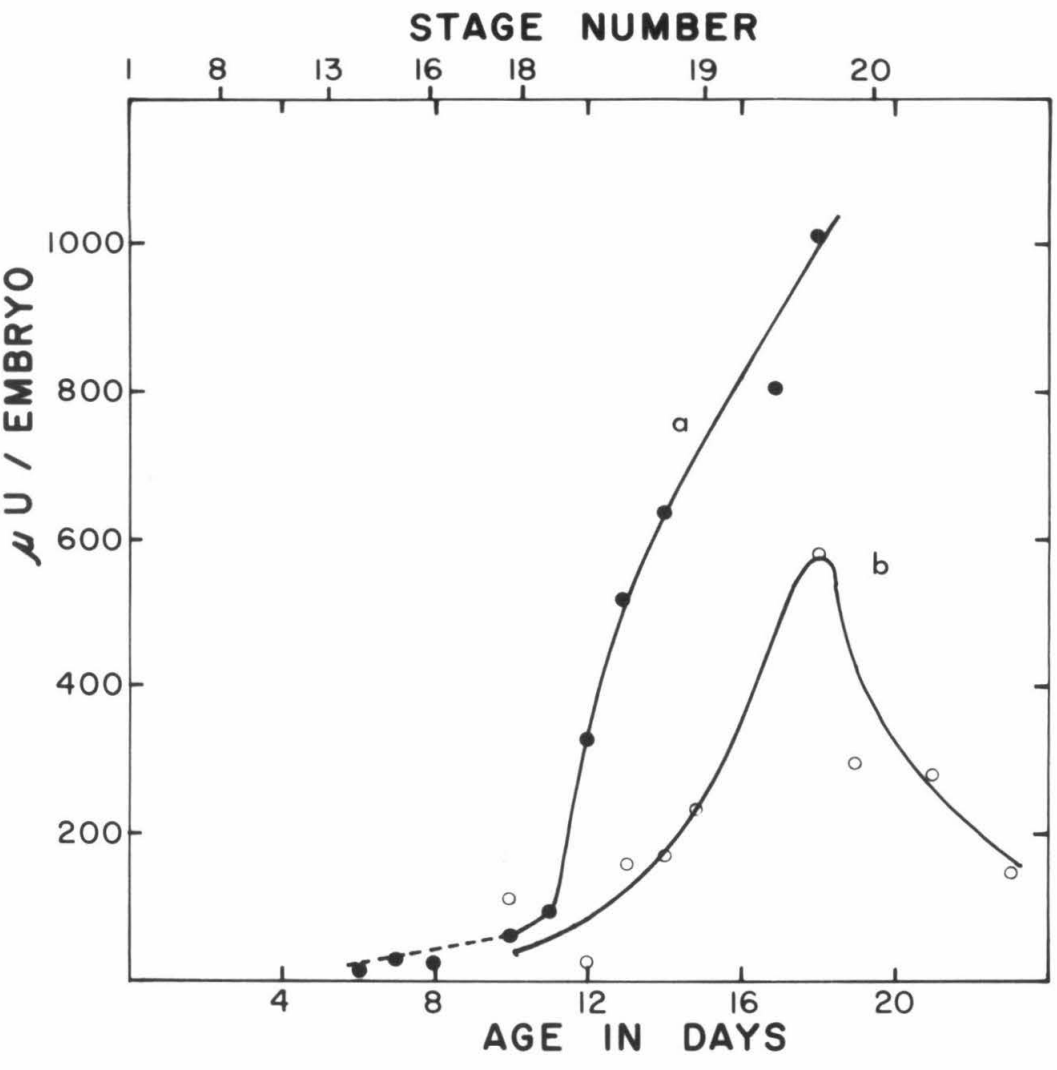


Fig. 3. Amounts of uricase in a) normal and b) unfed Rana temporaria embryos during the first 23 days of development (at 18°C), assayed by the photometric procedure detailed in the text. Each point represents the mean of duplicate determinations of an extract of between 5 and 20 embryos.

uricase synthesis. This hypothesis was tested negatively by starving a clutch, and changing their water frequently. No feces were observed from these animals. The results, shown in curve b, figure 3, show an initial rise sufficiently like the normal one that the timing would seem to be independent of feeding, until starving delays morphological growth and finally decreases the possibility for protein synthesis.

Thyroxin treated. The data which would carry figure 3, curve a, through metamorphosis are not plotted because they represent liver extracts only, and are not directly comparable with the other points, which represent whole embryos. These data show, however, that uricase activity continues to increase in premetamorphic and metamorphic stages.

It was attempted to test how closely the biochemical development of uricase was linked with physiological development, by truncating the premetamorphic period through the use of thyroxin. In a single trial, precocious metamorphosis was found to have no effect on uricase level unless perhaps to depress it somewhat.

Inhibitor treated. In order to test the hypothesis that sterically native enzyme is requisite to the synthesis of further molecules of that enzyme, embryos were raised in solutions of uricase inhibitors in sublethal concentrations, including cyanide, ascorbate, azide,

hydroxylamine, and xanthine. Some of the inhibitors were also injected into eggs or blastulae. No specific effects were noted, except that in one batch treated with 10^{-2} M xanthine until gastrulation, and 10^{-3} M thereafter, uricase assays read consistently five-fold lower than in control sibs. The latter single experiment would warrant repetition.

2. Organ Distribution

Uricase activity has in most studies with other animals been associated with the liver. Therefore in several experiments the liver was dissected out, with the pancreas and part of the duodenum to insure that the liver was removed in toto, and the liver was homogenized separately from the remaining parts. The activities of livers and remains are plotted, with simultaneous control assays of animals homogenized whole, in figure 4. Liver seems to account for slightly more than half of the uricase activity. A significant portion resides elsewhere, and was not traced further, except that intestine was excluded. Later experiments bearing on the point will be presented in part II.

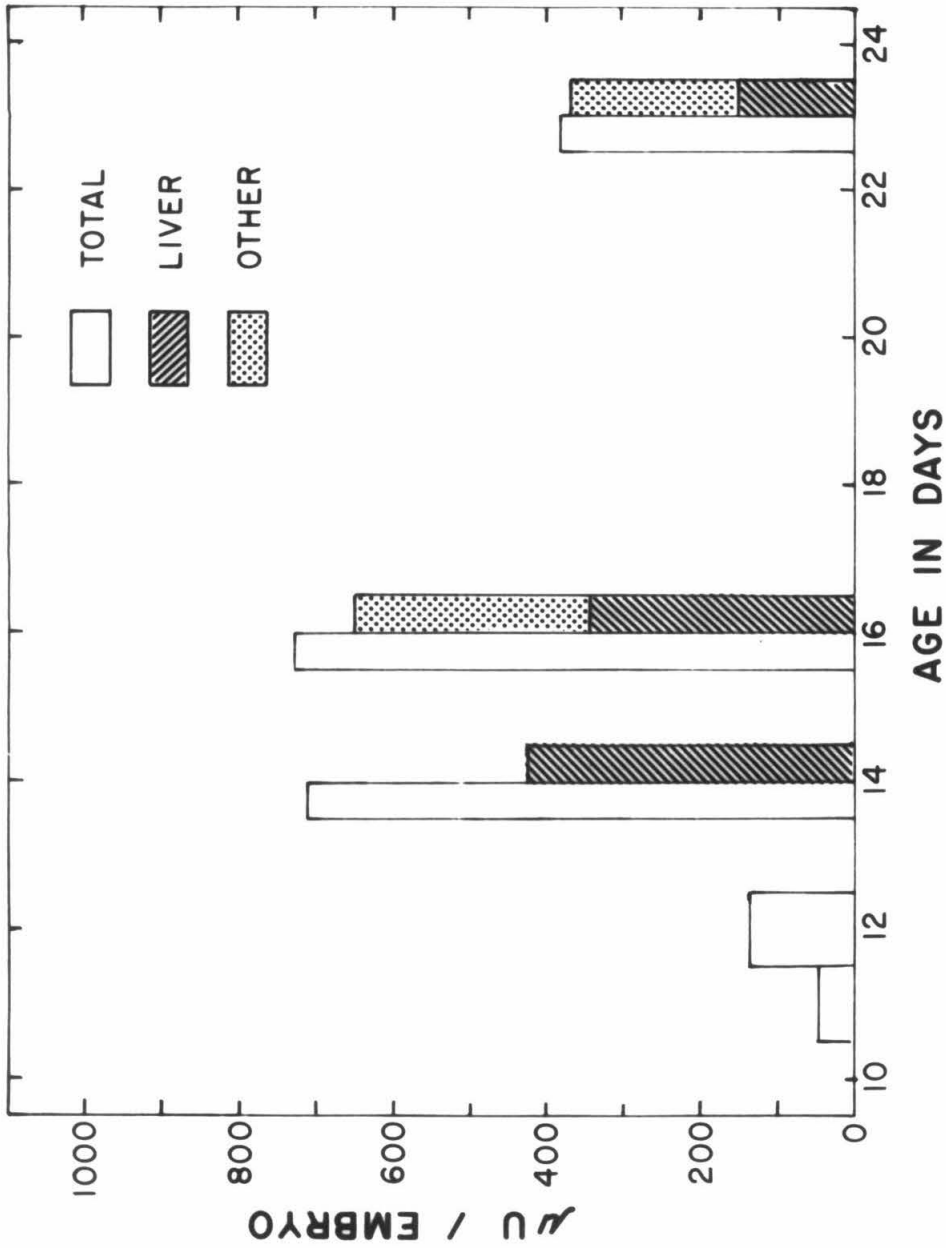


Fig. 4. The amount of uricase in early Rana temporaria larvae, and its partition between liver and all other organs taken together, determined at 14, 16, and 23 days of development by photometric assays.

D. Discussion

1. Enzymes Arising Contemporaneously

The discovery that uricase begins a major increase at stage 18 places it in the fifth of Løvtrup's categories (57), enzymes which reflect the functional differentiation of endodermal cells into the digestive tract. This is the period when liver and pancreas are structurally differentiating out of the liver diverticulum, and the gut is showing histological differentiation and lengthening, when yolk is rapidly disappearing from the cells and when, externally, the opercular fold is growing to cover the first pair of gills. Kopsch stage 18 (54) is equivalent to Shumway stage 23 and Harrison stages 43-46 (94) in other amphibian species.

In this class Løvtrup included amylase, trypsin, and alkaline phosphatase. Recent reports indicate that $NADH_2$-cytochrome c reductase (118) and other electron transport enzymes (55), and DNase I (20) also belong here. A literature search showed points of remarkable similarity among the five hydrolases, DNase I, alkaline phosphatase, α- and β-amylases, and alkaline proteinase. They are specialized secretions of the digestive glands, destined to act extracellularly at high pH, and three of them duplicate the activities of intracellular counterparts, more "general" enzymes, which arise earlier, operate best at a pH four units lower, are more generally

distributed among tissues, and are segregated in lysosomes. Alkaline phosphatase remains intracellular, however, in the intestinal mucosa, and the amylases do not have recognized intracellular doubles. It should be remarked that some of the assay methods were crude, and may well merge the activities of several similar species of enzyme.

The electron transport enzymes, $NADH_2$-, $NADPH_2$-, and succinate-cytochrome c reductases, as well as cytochrome oxidase, all show modest increases also at stage 18, according to Grant and Lang (55). Wallace, publishing at the same time (118), only partially confirmed these results. The disparity is difficult to explain, since essentially the same methods were used, but Wallace's values are lower, hence he would appear to have lost activity before or during assay in early points.

While the electron-transport enzymes are in a different class from the digestive enzymes, their increase can also be interpreted as a necessary adjustment to a new phase of activity, with more energy suddenly required and more energy sources available, through digestion. According to Witschi(125a), oxygen consumption increases eight-fold per embryo from stage 18 to 19.

2. Functional Need for Uricase. The reason for further oxidizing urate before excretion remains obscure, but the phase specific pattern of uricase ontogeny traced in these determinations suggests that the enzyme has functional significance related to the demands digestion makes of the organism. In the early embryo de novo synthesis of nucleic acids proceeds rapidly (39) and in gastrulae, purines down to the level of the free base can be utilized in nucleic acid anabolism (111). However, when feeding introduces fluctuating levels of purines (through nucleic acid digestion), it apparently becomes critical to the embryo a) that the purines be gotten rid of, and b) that in the process they be oxidized further than urate, despite the evidences which can be marshalled against this likelihood (p. 17).

3. Control of Uricase Increase

Two negative results were obtained in attempts to discern control mechanisms.

Although the accumulation of uricase would seem to be associated with the onset of the larva's digestive activities, experimental results do not support the idea that it occurs in response to ingested materials.

At stages past 20, enzyme concentration changes do not seem to be sensitive to thyroxin concentration or to the metamorphic

changes caused by that hormone. Uricase accumulation seems to follow a temporal rather than a physiological clock, at later larval stages.

PART II : RADIOISOTOPIC ASSAYS OF URICASE AND XANTHINE DEHYDROGENASE IN RANA CATESBEIANA

A. Introduction: New Methods

A simple order-of-magnitude calculation demonstrated the inadequacy of the spectrophotometric assay for a simple description of the amount of uricase present in anuran embryos.

Extracts from the earliest stages measured had all shown insignificant activity, to which a maximum value of 0.76 mU/ml could be assigned. The most concentrated of these extracts represented 0.014 mU/ embryo, or 1.4×10^{-11} mole S/min/embryo. Using Mahler's turnover number for purified porcine uricase (62) which, adjusted to the conditions of the assay used, was 2700, the calculation was:

$$< 1.4 \times 10^{-11} \frac{\text{mole S}}{\text{min. embryo}} \times \frac{6 \times 10^{23}}{2.7 \times 10^{3}} \frac{\frac{\text{molecules Enz}}{\text{mole Enz}}}{\frac{\text{mole S}}{\text{mole Enz} \cdot \text{min}}} =$$

$$< 3 \times 10^{9} \frac{\text{molecules Enz}}{\text{embryo}}$$

In the stages before uricase is detectable, the results allow the conclusion that between zero and three billion molecules of enzyme

are present per embryo. Nothing can be concluded of the shape of the broken portion of curve a, figure 3, before the tenth day.

Clearly before explanations of the mechanism of uricase regulation could be attempted, a new level of analytical accuracy was required.

This was found in a radioisotopic method, utilizing the selective labelling of the 6-C atom of the substrate, which is released as CO_2 in the overall reaction catalyzed by uricase (6).

Considering 0.015 O.D. units and 5 cpm above background as the lower significant response levels of the two methods, in 30 min assays, it was calculated that the isotopic assay is 85 times as sensitive as the photometric assay.

When preliminary trials showed that uricase was in fact present from the earliest stages, it was decided also to assay xanthine oxidase, the enzyme preceding uricase, and occupying a branch point, in the purine oxidative pathway (see fig. 2). Xanthine oxidase is usually present in tissues with uricase, but is reported in lower concentrations. It can be assayed by essentially the same radioisotopic method. The enzyme present in frogs was discovered to be actually the closely related enzyme xanthine dehydrogenase, requiring NAD as an acceptor. Despite the molecular similarity of the two enzymes (88), the systematic rules of the Commission on Enzymes (49) require that the classification stem

from the overall reaction catalyzed; thus the dehydrogenase may be designated "xanthine:NAD oxidoreductase," with a number yet to be assigned in sub-sub-class 1.2.1., while the oxidase, as "xanthine: O_2 oxidoreductase," has been assigned number 1.2.3.2.

With the expectation that comparison of activities of the two related enzymes in different organs might permit an attack on mechanisms of coordinate control, rough estimations of organ activities were also planned, at intervals in development.

Although the radio-assay does not require optical clarity, removal of small molecular weight contaminants from the crude enzyme preparation was still found to be necessary for reasons detailed below. In deference to the greater heat sensitivity of xanthine oxidase, dialysis was replaced by passage through a dextran gel column, which separated macromolecular components from small molecules within five minutes. The low M. W. fraction discarded after elution of the enzymatic fraction proved a useful starting point for determinations of endogenous substrates, in pursuit of an answer to the longstanding question whether substrate induction has any of the power in controlling the expression of metazoan genes that it has in microbial systems. The urate determinations are presented below, since they were performed on the catesbeiana specimens, although they do not depend on the isotopic method.

B. Methods and Materials

1. Biological Stocks

Rana catesbeiana, an introduced inhabitant of California, was chosen for continuing studies in Pasadena. While the species pipiens, on the basis of its early breeding season and low developmental optimal temperature, is probably more closely related to temporaria (125, p. 191), yet catesbeiana is within the same genus, and offers the advantages for biochemical work of much larger body size and egg masses. The two species are similar enough in morphology during development that Kopsch's finely detailed normal table for temporaria (54) could be used without difficulty for catesbeiana, with the exception of observations on the labial teeth of the larvae, which do not pertain (27). Similarly Brown and Cohen found a pipiens table readily applicable to catesbeiana development (10).

Stocks, presumably reasonably homogeneous through inbreeding, were obtained through Brescia's Frog Farm, Compton, California, from their breeding areas near Fresno, California. When attempts to induce ovulation in ripe females by injection of homospecific pituitaries failed, two clutches of naturally laid eggs were procured from the supplier, as well as samples of tadpoles of various ages past stage 20. The embryos were maintained at

18°C, the tadpoles at laboratory temperature, about 25°C, with sporadic additions of up to 0.1 mg/ml of Squibb Streptomycin, and up to 200 units/ml of Squibb Penicillin G. The larvae were fed with freshly boiled commercially frozen spinach.

Thus the true age of most of the embryos used in part II was unknown. Instead they were carefully staged before homogenization, and the abscissae of the developmental curves represent stage number. To make these results comparable to those of part I, however, the stage numbers were spaced unequally along the x-axis, according to the time spent in each stage by temporaria developing at 18°C. This adjustment renders the shape of curves in part I, where enzymatic activity is referred to age of temporaria embryos, roughly comparable to the shape of curves in part II, where activity is referred to the equivalent age of catesbeiana embryos. (To construct a graph of activities of catesbeiana larvae with their actual ages would be an arduous task: the wide variations in maturity which occur in catesbeiana of the same age, over the full year or even two years between their hatching and metamorphosis (27), are well recognized in the literature (126).)

2. Preparation of Extracts

Before initiating this second part of the work, a more careful study of extraction methods was undertaken, with the aim of finding a relatively crude procedure which would maximize the surviving activities of both enzymes. The general conclusion drawn was that the methods of the previous part were surprisingly near optimal, although dialysis was less suitable to the more labile xanthine dehydrogenase. All manipulations in this section were conducted at 0 to 4°C.

Release of bound enzyme. While xanthine dehydrogenase appears to be a soluble enzyme, uricase has long been recognized as particulate. Only recently, however, have the rat liver centrifugal fractionations of de Duve's group determined its exact location, in particles similar to (26), but not identical with (25), lysosomes. Lysosomes have been extensively investigated (81) since the pioneering work of the Louvain group, but uricase-containing particles remain largely ignored, and very little is known about them. Histochemically they are tentatively considered (81) equivalent to Rouiller's microbodies (91), i.e. like lysosomes they are delimited by a single membrane, but unlike lysosomes they are ferritin-poor and lamellated in internal structure. Physically they are identified by their behavior on sedimentation and particularly on

density-gradient centrifugation (25), and are considered anomalous in exhibiting no "opening phenomenon" (24). De Duve has found rat liver uricase activity to be the same before and after disruption of the particles, which apparently constitute no barrier to substrate access.

Preliminary experiments with gentle homogenization techniques (detailed in the appendix, p. 96) confirmed that in frog liver too, uricase is localized in the "light mitochondrial" (i.e. predominantly lysosomal) fraction (26), though the distinction between lysosomes and other particles was not pursued. Blending, freezing and thawing, salt solutions, and deoxycholate were efficacious in varying degrees in dislodging uricase activity from the 225K g-min-sedimentable, to the soluble, fraction, but no significant rise in activity was detected. It was concluded that assays would be valid whether the uricase were bound or free, so long as the conditions were standardized and the particles were included. This conclusion may come into question if repetition validates the results of a very recent experiment (preparatory to isolating ribosomes) which suggest that the demonstrable activity of uricase particles may indeed increase twofold on long incubation with deoxycholeate. As yet the discrepancy between this latter and previous results is unexplained.

Conditions of homogenization. Buffer at pH somewhat higher than 9.2 led to rapid coagulation of adult organ homogenates; several buffer systems at pH lower than 9.2 proved deleterious to xanthine dehydrogenase stability. Hence the 0.8 M Na glycinate buffer (pH 9.2) of part I was again used, as was homogenization by cone grinder, which freed about 50 per cent of the uricase. The next step, centrifugation at 30K g-min (2 min, starting from rest, in 8 X 43 mm tubes fitted in polyethylene adapters in the 295 head on the multi-speed attachment of the International Refrigerated Centrifuge, Model PR 2, set for 19K rpm), sedimented cell debris and nuclei, clarifying the homogenate without removing from the supernatant the one half of uricase remaining particle-bound.

Gel filtration. In order to assure reproducibility and low background in the radioisotopic assay, it was necessary to rid the crude supernatant of possible low molecular weight contaminants of at least four sorts: 1) substrate, which would dilute the radioactivity of the urate-6-C^{14} to be added; 2) inhibitors of both enzymatic reactions to be assayed; 3) non-enzymatic catalysts which can accomplish the same reactions; 4) small molecules which would act as substrates for enzymatic fixation of $C^{14}O_2$ released by uricase action, into compounds which would not be released by H_2SO_4 for trapping and counting. Consideration of at least the first three sorts of contaminant was of more than theoretical interest.

A sample of crude liver homogenate added to a dialyzed extract actually decreased the $C^{14}O_2$ released from urate by 59 per cent, and naturally occurring xanthine oxidase inhibitors are mentioned in the literature (8). Evidence for the presence of substrate and non-enzymatic catalysts of the uricase reaction is presented elsewhere in this thesis.

The unique rapid filtering properties of Sephadex, a cross-linked dextran, were utilized in place of dialysis for this step. Separation on a Sephadex column proceeds by partition of a solute between the solvent of the outer volume, V_o, external to the gel particles, and the solvent of the inner volume, V_i, comprising the interstices in the gel granules (83). A substance is characterized by its coefficient of partition between the two solvent phases, K_D, derived from the flow equation

$$V_e = V_o + K_D V_i ,$$

where V_e designates the volume passing through the column between application and elution of the substance (34). Macromolecules are excluded by their size from entering the inner volume, hence $K_D = 0$, whereas small molecules must pass also through the interstitial volume before eluting, so that their $K_D = 1$ theoretically, reduced to 0.8 in practice by fixed internal water of hydration.

Dry Sephadex G-50, 50-270 mesh, with a water regain of 5.2 g per g of dry material, was obtained from Pharmacia, Uppsala, Sweden, and allowed to swell in 0.1 M Na glycinate (pH 9.2). It was freed of fine particles by several decantations, and packed in two identical columns of 9.5 mm inner diameter and 32 mm height. These two columns were used repeatedly, as the gel automatically regenerates in each separation process. The filter paper disc covering the top of the column to prevent disturbances was replaced frequently. Flow through the columns was by gravity without added pressure. Recovery of the enzyme from the column was accomplished within five minutes in most runs. All separations were conducted at 4°C. Uricase-containing particles flowed through without hindrance, as was expected, since various viruses do so (67).

In calibrating the columns, V_o was taken as the volume of buffer required to pass a methemoglobin solution down the column. V_i was calculated from the volume and density of the gel, and the water regain value was furnished by the supplier. Commercial uricase, xanthine and urate solutions in 0.8 M glycine buffer were applied to one column, and their elution by 0.1 M buffer followed by spectrophotometric measurements. The elution histograms are shown in figure 5. The enzyme eluted with a K_D of 0, as expected, and both substrates together, with K_D about 0.8 (only urate is graphed).

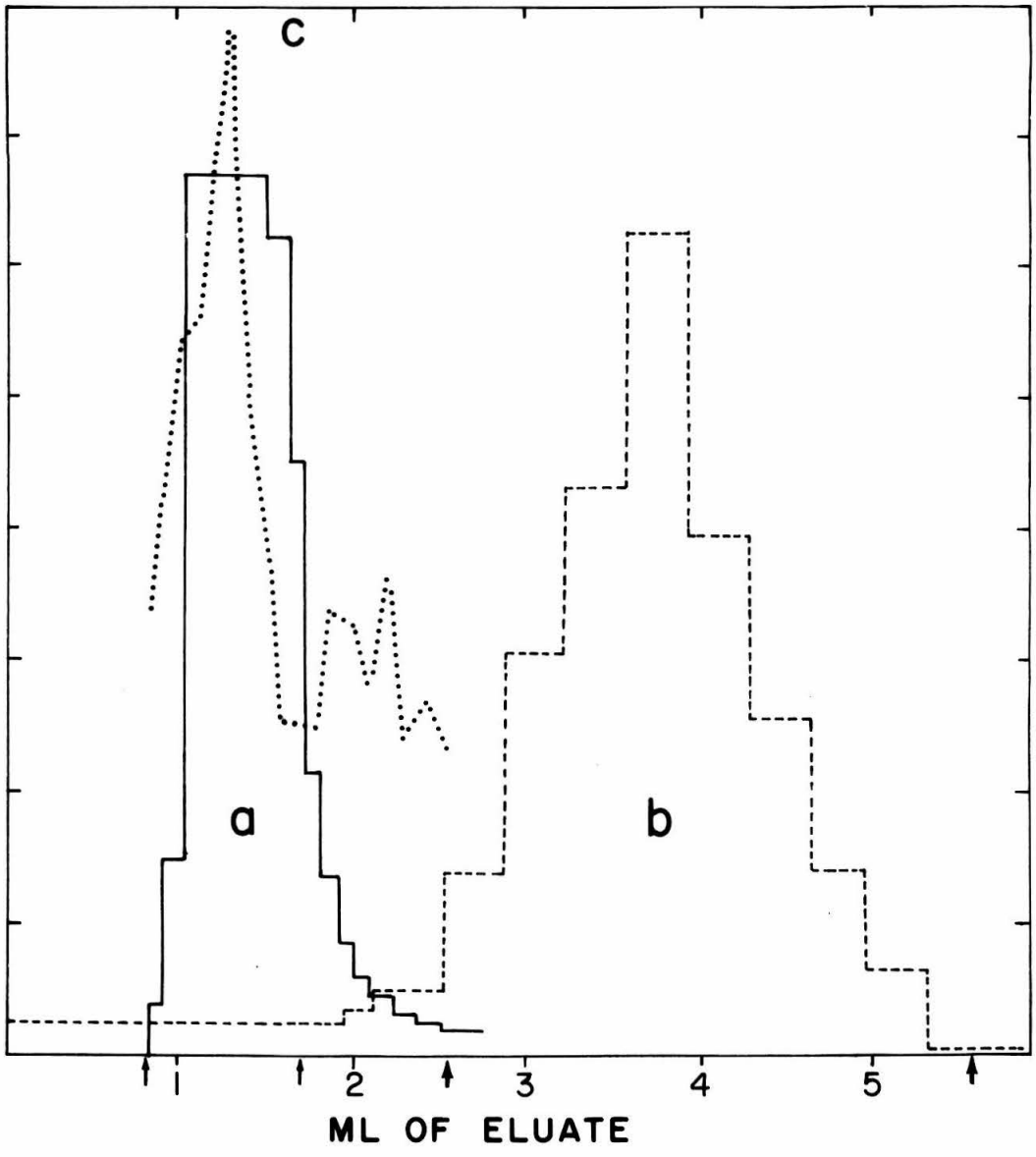


Fig. 5. Elution from a Sephadex G-50 column by 0.1M Na Glycinate at pH 9.2 of a) Worthington purified uricase, assayed as in Methods, part I; b) Na H Urate, read as O.D. at 292.5 mμ; and c) urate oxidizing capacity of an embryonic extract, determined by radioisotopic assay. The ordinates are arbitrary units. Arrows delimit the fraction routinely collected as enzyme, bold arrows the fraction taken as urate.

Subsequently an actual embryonic extract was gel filtered, and the eluate fractions incubated with urate-6-C^{14} for determination of $C^{14}O_2$ released. Results are pictured in curve c of figure 5. Clearly, besides the enzyme, another component with K_D about 0.6 is effecting urate oxidation, and perhaps a third. Partial exclusion of small molecules, so that K_D values fall below 0.8, has precedent in the literature, particularly with alkaline eluents (34), and indeed a brief experiment showed that Cu^{2+}, one well-known catalyst of urate oxidation (41), would elute in this range. The limits of the fraction routinely to be taken as enzyme were specified to exclude these components.

However, either a small part of the lead edge of such a component, or perhaps ions adsorbed to macromolecules, would appear to accompany the enzyme fraction after gel filtration, since gel filtered samples of several adult organs showed residual uricolytic power after boiling. The non-enzymatic reaction did not represent more than ten per cent of the total activity in the low-activity organs studied (where the error introduced would be most serious), including lung, intestine, and pancreas. Correction for the error was not attempted. No significant uricolytic activity remained in a boiled embryonic extract, and xanthine dehydrogenase activity was not found in boiled samples of embryonic or adult materials.

Figure 5 also illustrates that the substrates for the two enzymes of interest could be obtained almost enzyme-free as a by-product of the Sephadex filtration, in a 3 ml sample collected about 0.5 ml after the enzyme fraction. Such samples were retained in some runs as the starting point for urate determinations, see section 6 below.

3. Uricase Assay

When trapping the $C^{14}O_2$ released by uricase action in hyamine hydroxide by means of a helium train proved unreproducible as well as slow, it was decided to use closed flasks for both the reaction and the trapping, and a method was patterned after that of Ultmann and coworkers for xanthine oxidase, which was already in the literature (115). Reagents were made up with special precautions against cationic contaminants, to which urate seems to be more sensitive than xanthine (41).

Protocol. 1. Place the reaction mixture minus substrate in the main compartment of a Warburg flask, including a gel filtered sample to be assayed, and 0.1 M Na glycinate (pH 9.2), to make 0.490 ml. The buffer is made by bringing 0.8 M glycine (Nutritional Biochemicals Corporation, Cleveland) to pH 9.2 with 6 N NaOH, and passing the solution over Dowex 50 (Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.), collecting the eluate

after pH reaches 9.2.

2. Place 0.4 ml of a mixture of 1 toluene : 1 Hyamine hydroxide (1 M solution in methanol, obtained from Packard Instrument Co., LaGrange, Ill.) in the center well, using a glass chimney to avoid any drop of the strong quaternary base's contaminating the main compartment. (Hyamine is insoluble in water, so that any major contamination of Hyamine by water or vice versa is apparent as a white precipitate).

3. Place approximately 0.4 ml. of 6N H_2SO_4 (Reagent Grade, E. I. du Pont de Nemours and Co., Wilmington, Del.), which has been passed through Dowex 50 to remove metallic impurities, in the sidearm, and stopper it.

4. Shake the flask briefly in the Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago) to equilibrate with the bath temperature, 30°C.

5. At t = 0, pipet 0.010 ml of substrate into the flask to start the reaction. Quickly stopper the flask and return it to the incubator, shaking at 110 strokes/min. The substrate is 3.4×10^{-3} M NaH Urate-6-C^{14} at 0.432 mc/mmole; the radioactive product supplied by Isotopes Specialties Corporation, Burbank, California, is purified on a Dowex 1-formate column as detailed on p. 59 below, diluted tenfold with cold NaH urate from Pfanstiehl Laboratories, Inc., Waukegan, Illinois, and stored frozen in small

aliquots until immediately before use. The reaction mixture, then, is 6.8×10^{-5} M in urate, with 1.68×10^{4} cpm.

6. At t = 30 min, tip the H_2SO_4 from the side-arm into the main compartment, stopping the enzymatic reaction and releasing the labelled CO_2. Continue shaking.

7. At t = 60 min, quantitatively transfer the Hyamine from the center well to a 20 ml low-K^{40} counting vial, using 19 ml of scintillator mixture prepared as follows:

2 l toluene

6.5 g "PPO" (2,5-diphenyloxazole, scintillation grade, Packard)

0.65 g "POPOP" (1,4-bis-2-(5-phenyloxazolyl)-benzene, scintillation grade, Packard Instrument Co.)

0.5 l 100% ethanol

8. Count C^{14} activity by liquid scintillation spectrometry. A counting efficiency of 47.5 per cent was achieved on a Tri-Carb Spectrometer Model 314-EX (Packard Instrument Co.), using high voltage setting 4.00.00.

9. Wash all glassware with hot water and detergent, rinse with distilled water and then deionized water. Attention should be drawn to the use of "K-Y Sterile Lubricant" (Johnson and Johnson, New Brunswick, N. J.) for sealing the ground

glass stoppers on the Warburg flasks. This water-soluble lubricant obviates the time-consuming procedures required to cleanse the flasks of greasy seals (116), and frees them for quick re-use.

As facility was acquired, determinations were run with as many as 16 flasks in tandem, the times being staggered 30 sec apart.

Calculations. The calculation of μmoles of CO_2 released by an enzyme aliquot per minute at 30°C and pH 9.2, and extrapolated to infinite substrate concentration, is straightforward. The correction to maximal substrate is

$$\frac{V}{v} = 1 + \frac{K_M}{[s]} = 1 + \frac{4.3 \times 10^{-5}}{6.8 \times 10^{-5}} = 1.63$$

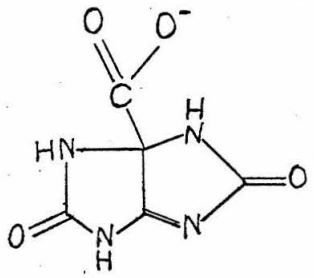
The specific radioactivity of the C^{14}, determined by comparing radioactivity of the substrate to its O.D. at 292.5 mμ, was 4.93×10^5 cpm/μmole.

Thus,

$$1 \frac{\mu mole}{min} \times \frac{30 \text{ min}}{1.63} \times 4.93 \times 10^5 \frac{cpm}{\mu mole} = 9.06 \times 10^6 \text{ cpm.}$$

The international unit, however, is defined in terms of substrate transformed, rather than product formed (p. 21). To equate μmole/min, as calculated above, to enzyme units rests on the assumption of a stoichiometric relationship between urate loss and CO_2 formation. This assumption is invalid.

Schuler first reported (97) that release of CO_2 is not equivalent to uptake of O_2, and asserted that the hydrolytic portion of the reaction is non-enzymatic, and distinct from the initial enzymatic oxidation. Mahler has recently concluded an intensive investigation of the action of uricase, and has confirmed this separation. According to his scheme (61), the initial enzymatic oxidation leads only as far as "intermediate I," which he pictures as:



The compound is strained, and would apparently suffer immediate decarboxylation. If on the other hand intermediate I were stabilized by the milieu, decarboxylation might be rate-limiting, and account for the non-stoichiometry. Hübscher, Baum, and Mahler earlier (49) stressed the importance of the environment in determining products of this reaction; clearly each case must be considered separately.

That the isotopic assay yields internally consistent results is demonstrated in the next section, and argues that the efficiency of release of CO_2 from intermediate I is a constant under the conditions used. This constant was evaluated as 36.4 per cent, and

applied to the raw calculation to yield a conversion factor to the international unit:

$$1\ U = 9.06 \times 10^{6}\ \text{cpm} \times 0.0364 = 3.30 \times 10^{6}\ \text{cpm}$$

The few determinations performed before the conditions given here had been worked out have been normalized to compare with determinations which followed the standard protocol.

Standard errors were calculated by the following formula (82) in cases where the gross counts were fairly close to background level:

$$\%\ \text{S.E. in net count} = \frac{100}{\sqrt{N}} \left[\frac{1 + \frac{B}{R}}{\left(1 - \frac{B}{R}\right)^{2}} \right]^{\frac{1}{2}}$$

where N = total number of counts accumulated

B = background cpm

R = gross cpm

Linearity. The $C^{14}O_2$ counted was found linearly proportional to the amount of enzyme present, to at least a total of 2000 cpm, within which range all the experimental determinations fell. The plot is shown in figure 6.

The isotopic product was proportional to incubation time well past 30 min, as figure 7 illustrates.

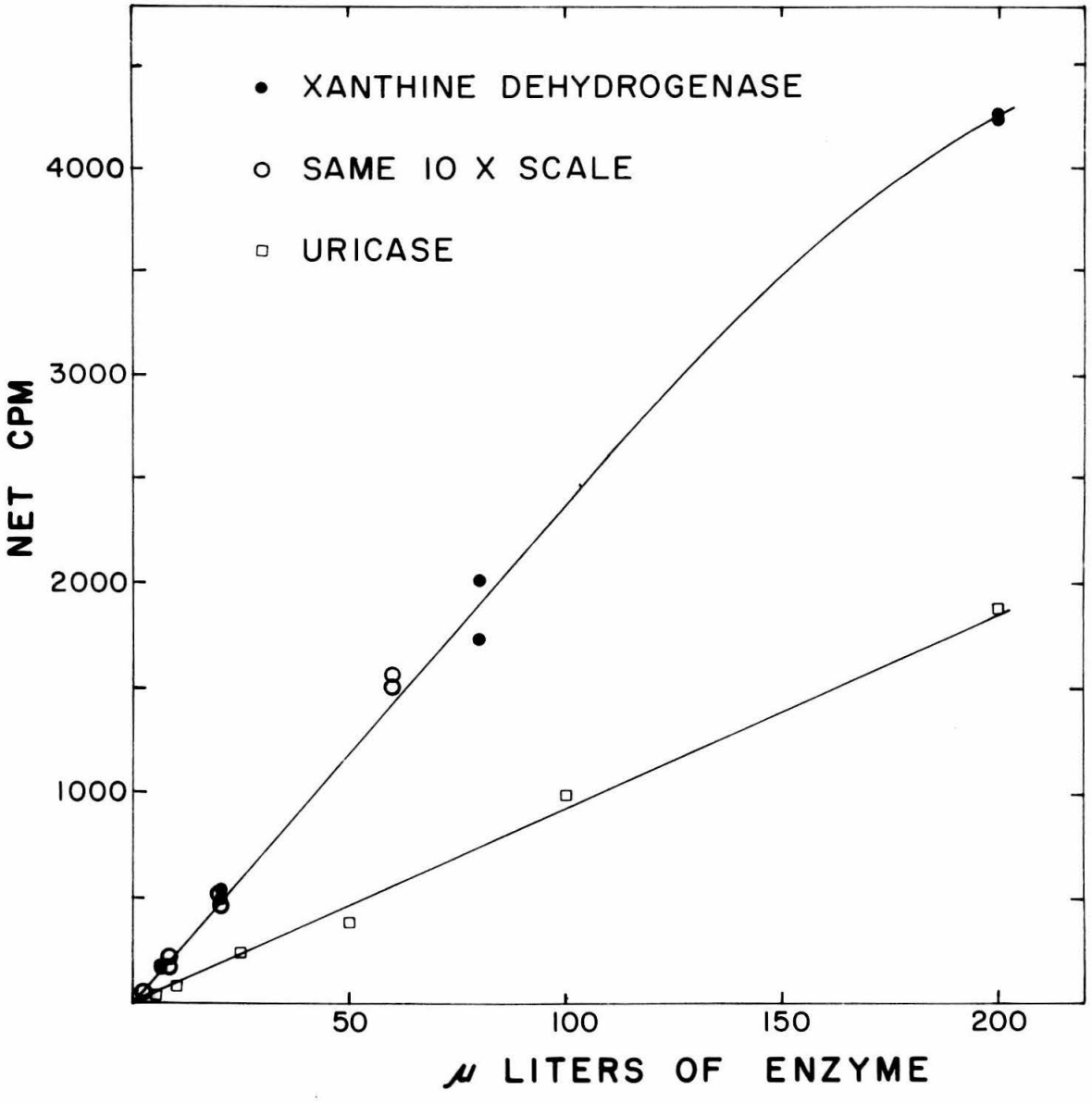


Fig. 6. Radioisotopic assays of uricase and xanthine dehydrogenase: relationship between CO_2 produced and amount of enzyme used. The procedures are given in the text. Low xanthine dehydrogenase points are replotted with ten-fold expanded axes to show constant slope at low activities.

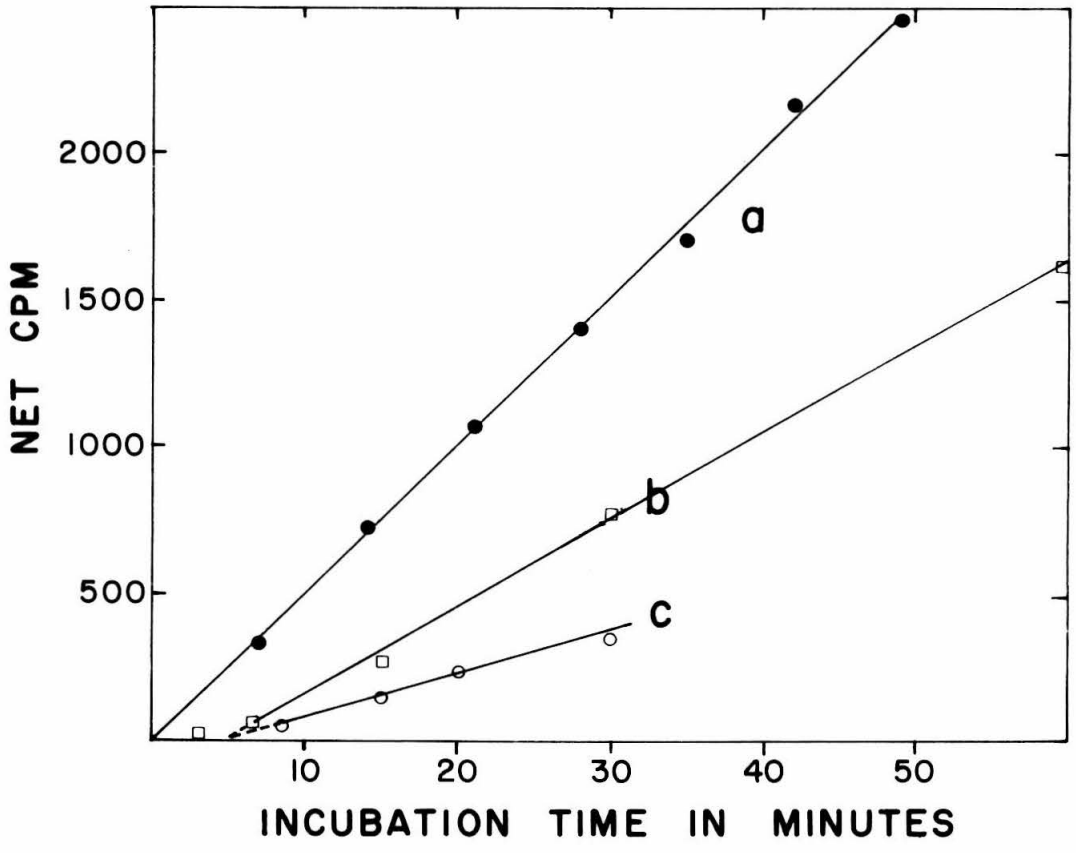


Fig. 7. Radioisotopic assays of uricase and xanthine dehydrogenase: linear relationship between CO_2 produced and time of incubation. Aliquots of one uricase sample (a) and two different samples of xanthine dehydrogenase (b, c) were used.

4. Xanthine Dehydrogenase Assay

With 6-C^{14}-labelled xanthine for substrate, and an excess of exogenous uricase, the method for determining uricase is applicable to xanthine dehydrogenase. The method of Ultmann, Feigelson, and Harris (115) was followed where possible.

Cofactors. In some preliminary experiments a small stimulation of xanthine oxidase activity by FAD (4×10^{-4} M) was noted, but the factor of increase in non-enzymatic substrate degradation was of the same magnitude. The effect of adding flavin was neither reproducible enough nor profitable enough (in terms of sensitivity) to be adopted routinely. FAD in the assay mixture was equally stimulatory to enzyme preparations whether they had been pre-incubated with FAD before gel filtration or not, suggesting that the effect was exerted externally to the one FAD moiety bound in each molecule of holoenzyme (88) in both dehydrogenase and oxidase.

When NAD ("DPN," Boehringer u. Soehne, GMBH, Mannheim) was added, to the extent of 1.2mM , the increase in CO_2 released was striking, amounting to a 39-fold stimulation of a gel filtered enzyme preparation, 47-fold stimulation of a charcoal treated preparation. This was taken as an indication that a dehydrogenase was present rather than, or perhaps in addition to, an oxidase, in accordance with the criteria of Remy et al. (88), Mahler (60), and

Glassman and Mitchell (37). (The same concentration of NAD, by contrast, stimulated milk xanthine oxidase only 20 per cent.) Activities in the absence of exogenous acceptor were attributable to lingering traces of cofactor after gel filtration, but it could not be excluded that a small part of the activity was a true oxidase, able to transfer electrons to O_2. In one experiment, however, a competitive inhibitor of NAD, 3-pyridine-aldehyde-NAD (B grade, California Corp. for Biochemical Research, Los Angeles) reduced activity of a Sephadex-treated extract by 15-fold, nearly to background, while NAD increased its activity 30-fold to 1000 cpm.

"Antabuse" (tetraethyl thiuram disulfide, from K and K Laboratories, Inc., Jamaica 33, N. Y., recrystallized twice from ethanol), which inhibits the activity of mammalian liver xanthine oxidase (89), but not avian liver dehydrogenase (87), was ineffectual against frog enzyme, adding to the evidence for its dehydrogenase nature.

The enzyme exhibited acceptor specificity, activating only 4-fold in the presence of 1.2 m$\underline{M}$ NADP ("TPN," California Corp. for Biochemical Research).

In contrast to methylene blue, which raised substrate blanks considerably, NAD had no effect on the substrate in the absence of enzyme.

Protocol. The schedule for xanthine dehydrogenase differed only in reaction mixture from that for uricase (p. 42), and flasks of the two were often run together.

The contents of the main compartment of the flask were:

0.010 ml	purified uricase, about 12mU (Worthington Biochemical Corp.)
0.010 ml	6×10^{-2} M NAD in the pyrophosphate buffer. Enzyme sample, gel filtered, in 0.1 M Na glycinate.
0.1 M	Na pyrophosphate (pH 8.0), to make
0.490 ml	total
0.010 ml	4.45×10^{-3} M xanthine-6-C^{14} at 1.22 mc/mmole (as supplied by Isotopes Specialities Co., with sufficient NaOH to dissolve)

Substrate was added last to start the reaction. The reaction mixture was 8.9×10^{-5} M in xanthine, with 6.18×10^{4} cpm.

Calculations. The xanthine dehydrogenase unit (U) is defined, in accordance with the report of the Commission on Enzymes (49), as that amount which will catalyze the transformation of 1 μmole of xanthine per minute at 30°C and pH 8.0, extrapolated to infinite substrate concentration.

The calculations for CO_2 formation are analogous to those for uricase:

$$\frac{V}{v} = 1 + \frac{K_M}{[s]} = 1 + \frac{6.7 \times 10^{-6}}{8.9 \times 10^{-5}} = 1.08$$

$$1 \frac{\mu mole}{min} \times \frac{30 \text{ min}}{1.08} \times 1.39 \times \frac{10^6 cpm}{\mu mole} = 3.73 \times 10^7 \text{ cpm}$$

The factor relating substrate loss to product formation was expected to reflect the subtheoretical yield of CO_2 from urate, reported above as 36.4 per cent. But when the factor was empirically determined, one mole of xanthine was found to yield only 0.084 moles of CO_2 under the conditions used. Photometric evidence at hand indicated that xanthine is quantitatively converted as far as urate, so that the loss was attributable to either inefficient attack of the added pure uricase on the sub-nanomolar concentrations of urate produced, or less efficient breakdown of intermediate I under these altered conditions, or a combination of both effects.

Suggestive evidence implicates at least the former effect. Figure 7 shows that although the incubation time routinely used is well within the linear portion of a plot of CO_2 formed versus time of incubation, the lower part of that curve extrapolates to a five minute lag period. This finding prompted recognition that although the

uricase appears to be in at least 100-fold excess over the largest xanthine dehydrogenase activities determined, actually the effective concentration is somewhat lower, as the xanthine present as substrate is a uricase inhibitor with $K_i = 1.2 \times 10^{-5}$ M (Mahler (61), confirmed in this laboratory as 1.1×10^{-5} M).

Thus it was expected that the uricase-mediated reaction might be less efficient at lower than at higher xanthine dehydrogenase activities, and the proportionality of CO_2 production to amount of dehydrogenase was carefully investigated down to the lower limit of significance of the method. Figure 6 clearly shows that linearity obtains from 0 to 2000 cpm. Above this level the slope decreases slightly, presumably due to xanthine depletion, and the very few higher experimental values had to be corrected by interpolation.

The conversion factor then, can be applied with confidence to all activities found, to yield the unit based on substrate loss:

$$1 \text{ U} = 3.73 \times 10^{7} \text{ cpm} \times 0.084 = 3.13 \times 10^{6} \text{ cpm}$$

Ultmann's data (115) were subjected to closer scrutiny, inasmuch as the conditions for these assays closely followed his. The four points of his time curve were found to fit better to a plot through 7 min than to that drawn through the origin. The implied dependence on uricase throws doubt on his Lineweaver-Burk plots. The proportionality of CO_2 to enzyme which, as demonstrated, is

valid for constant xanthine concentration, may well be questioned when xanthine varies 20-fold, so that K_M for uricase is multiplied between two-fold and 20-fold.

Michaelis-Menten Constant. A frog liver extract was used for a determination of the K_M of xanthine dehydrogenase, drawing fully on the sensitivity of the isotopic method. The details of the standard procedure were followed, except that the substrate was varied from 9×10^{-7} M to 7×10^{-6} M, so as not to inhibit uricase singificantly. A low enough enzyme activity was employed to avoid errors of substrate depletion. The K_M so derived was 6.7×10^{-6} M. The Lineweaver-Burk plot is shown in figure 8.

5. Protein Determinations

Protein content of gel filtered enzyme preparations for specific activity calculations were determined by the Ellman modification of the biuret reaction, measuring the difference in absorbance at 263 mμ between alkaline protein solutions with and without copper ion (29). Ellman's procedure A was followed, at one-sixth scale, so that final volume of reaction mixtures was 0.55 ml.

Proteins differ in the vigor of color formation with the reagent, thus a protein or mixture of proteins is characterized by the slope of an O.D. versus concentration plot, $\Delta A/(mg/ml)$ cm.

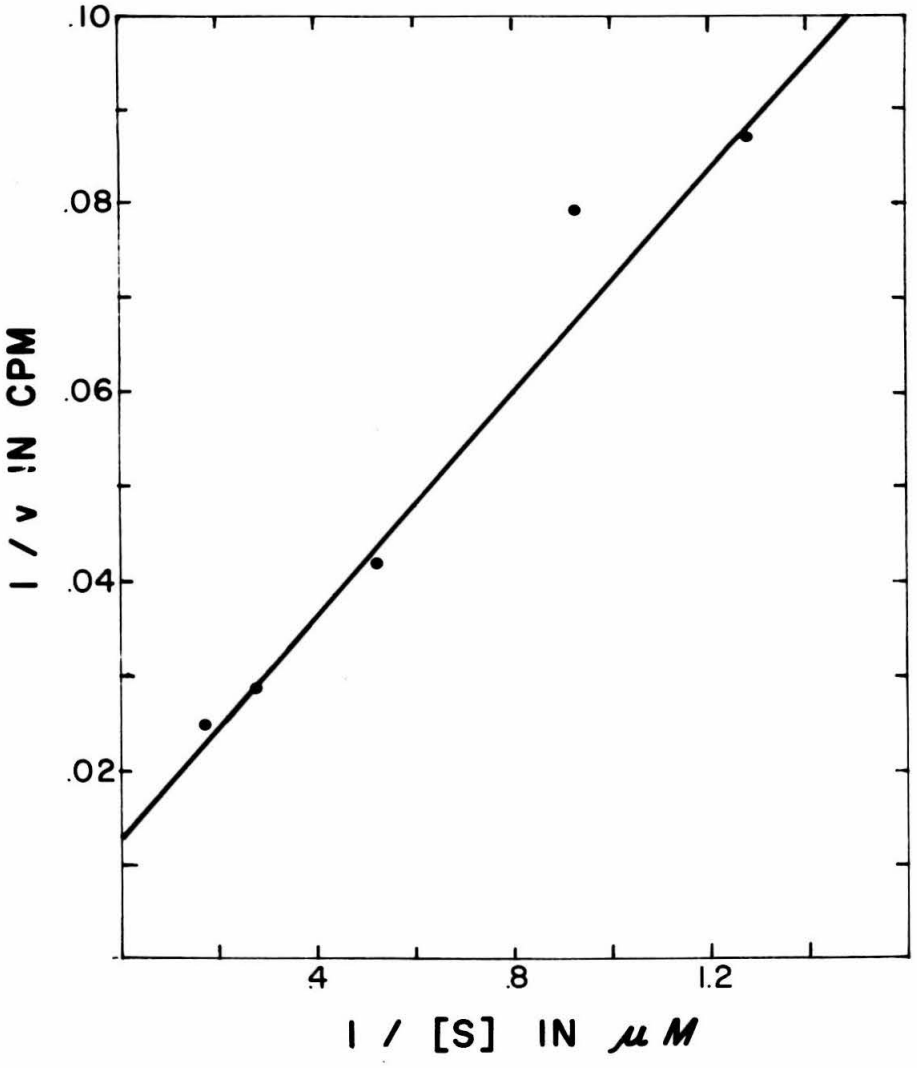


Fig. 8. Lineweaver-Burk plot for the determination of the Michaelis-Menten constant of Rana catesbeiana larval liver xanthine dehydrogenase, under very low substrate concentrations, with NAD as acceptor. The procedure varied only in substrate concentrations from that described in part II Methods.

The system was tested with a pure protein, bovine serum albumin, and a slope of 5.4 was found, in good agreement with Ellman's 5.3 (29).

Protein was then purified from tadpoles of about stage 20, so that it could be weighed and the system calibrated. The slope given by tadpole protein was 5.3, see figure 9.

The protein was purified as follows:

1. Homogenize 16 mm tadpoles in water. Add 1/10 volume 20% trichloroacetic acid (TCA). Centrifuge at 30K g-min.

2. Dissolve ppt in 1N NaOH, recentrifuge. Make supt 10% in TCA. Heat to 84°C for 10 min. Centrifuge.

3. Repeat step 2 twice.

4. Wash the ppt, homogenizing and centrifuging each time, twice with 1 ethanol: 1 diethyl ether; twice with 1 ethanol: 1 chloroform; three times with ether.

5. Dry in a desiccator.

Protein concentrations were routinely determined graphically from assays of at least two different volumes for each sample.

6. Assay of Urate in Embryonic Extracts

Attempts to determine the amount of urate present in crude homogenates were begun with Wharton's $HgAc_2$-HAc precipitation

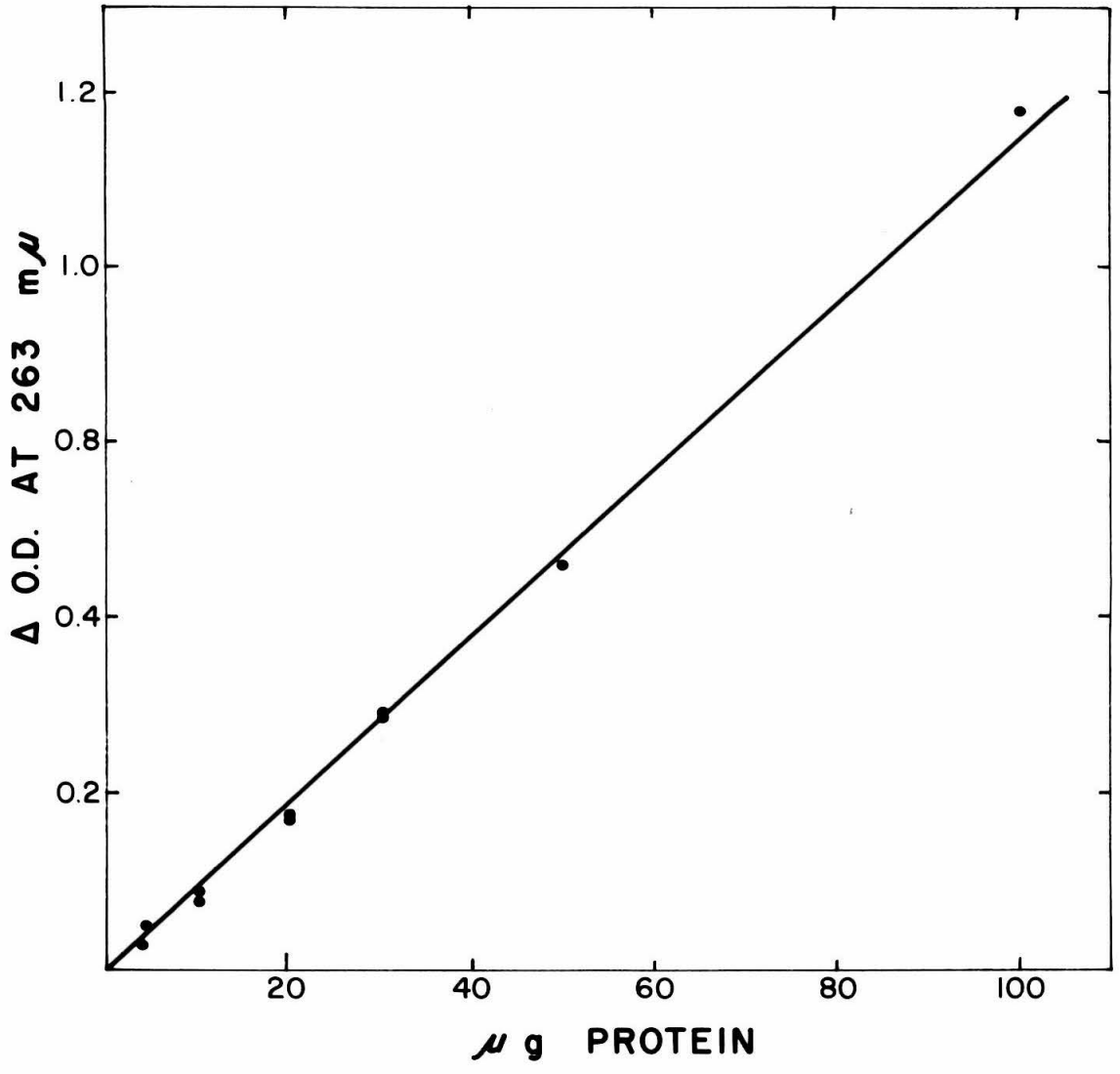


Fig. 9. Standard curve for the determination of Rana catesbeiana protein by the method of Ellman (29). Difference in optical density at 263 mμ between alkaline protein solutions with and without Cu^{2+} is plotted against μg of protein per 0.55 ml reaction mixture. Protein purified from whole larvae was used as the standard.

method (122), which proved completely unsatisfactory for this material. For acceptable specificity the method of choice seemed to entail following the decrease of absorbance at 292.5 mμ after adding purified uricase.

A preliminary experiment, however, showed that less than 3 per cent of the diffusate (after dialysis) absorbing at 292.5 mμ was vulnerable to uricase, hence the need for enrichment of urate in the small-molecular fraction was clear. That purines of biological occurrence (e.g. xanthine) are known to inhibit uricase action, constituted another urgent reason for partially purifying the urate before assaying it enzymatically. It was apparent that the sharp separations attained with Dowex chromatography (13) could be well applied to the problem.

Columns. Dowex-1-formate was prepared by the method of Hurlbert et al. (48) and poured into several identical miniature columns of 5 mm inner diameter and 1.2 ml bed volume, fitted at the top with wide reservoirs.

Application. Immediately after collection, the low-molecular weight portion of the Sephadex eluate (indicated by double arrows in fig. 5) of an embryonic homogenate was applied to the Dowex column in the cold, 15 ml of distilled water were washed through to remove any trace contaminant of endogenous uricase, and the column was clamped off, covered, and stored in the cold.

Elution. When all six columns were thus charged, stepwise elutions were conducted in parallel, at room temperature. The columns were permitted to go dry briefly before each addition so that eluent steps were distinct. Elution by ammonium formate (AmF) at pH 5.1, followed this schedule:

Add	5.0 ml	0.1M AmF	Discard
	2.5 ml	0.2M AmF	Discard
	5.0 ml	0.2M AmF	Collect as "xanthine"
	4.0 ml	0.5M AmF	Discard
	5.0 ml	1.0M AmF	Collect as "urate"

The columns were regenerated for re-use (48).

Evaporation of Eluent. The 5.0 ml eluate fractions were evaporated to dryness and the AmF (which would interfere with the enzymatic determination) evaporated off, by successive lyophilization and vacuum evaporation at 60°C. The lyophilization step was introduced when urate was found unstable to slow evaporation in vacuo at room temperature (as is suggested by observations in the literature (2,41,66)).

Enzymatic Determination. The dry sample was dissolved in 1.0 ml of 0.1M Na glycinate (pH 9.2). Within 15 sec of the addition of about 3 mU of purified uricase (Worthington Biochemical Corp.), the solution was thoroughly mixed and an initial O.D. reading was

obtained in a Cary Model 11 Recording UV Spectrophotometer. After incubation, corked, at 30°C for approximately 10 hr , the solution was read again. Control mixtures lacking enzyme were used to make minor corrections for evaporation and machine drift.

Calculations. Urate concentration was calculated using as the millimolar extinction coefficient 11.6, the published value (14), which has been confirmed under the conditions of the present work. Calibration curves showed better than 95 per cent recovery of urate during the evaporation and determination stages. The loss during chromatography may reach 10 per cent, but was not corrected for.

Xanthine. Xanthine is cleanly separated from urate as the elution scheme suggests, and the method could easily be extended to include enzymatic xanthine determinations from the same original samples.

C. Results

1. Enzyme Activities of Whole Embryos

Uricase. The data on animals from two clutches are combined in figure 10. Each point represents at least two assays of one embryonic extract. The abscissae are subject to fairly substantial error, as explained in Methods and Materials above, since they could be classified only to stage, and, particularly past stage 16, stages are often of several days' duration. The break at stage 18, where rapid accumulation begins, and the fact that the enzyme was at all stages well within the detectable range are salient points. Contamination of the extracts by non-enzymatic catalysts would not appear to be a problem, since determinations on one of the least active enzyme preparations set a higher limit of five per cent on the activity resistant to boiling.

Xanthine Dehydrogenase. The xanthine dehydrogenase levels for the same samples are presented in figure 11. A similar, though apparently sharper, break is noted, probably occurring late in stage 18. Although xanthine-6-C^{14} was used without carrier dilution, its low conversion to CO_2 renders the xanthine dehydrogenase assay about equally sensitive to the uricase assay. This detection level was not quite adequate for the early embryos with low activity, and the three lowest points in figure 11 actually differ from

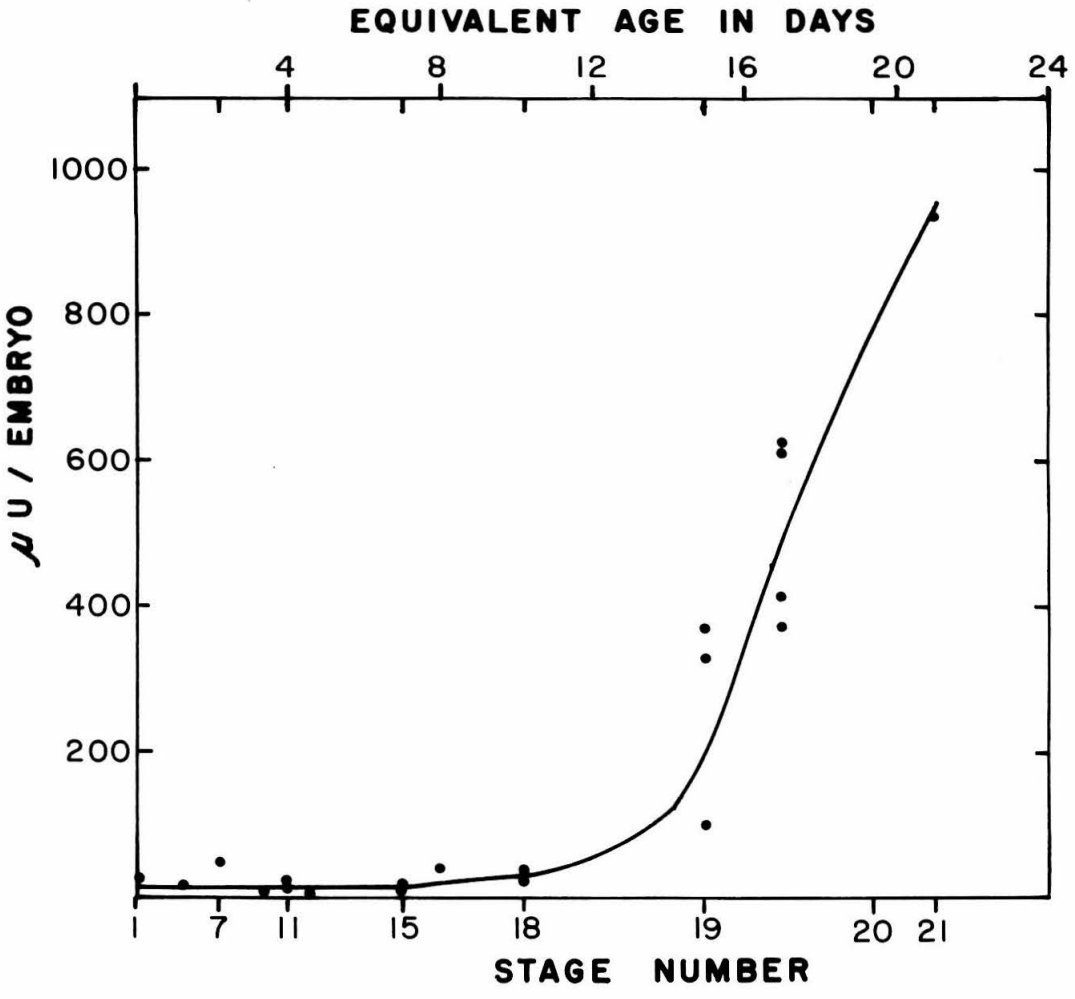


Fig. 10. Amount of uricase in normal Rana catesbeiana embryos during development to stage 21, assayed by the radioisotopic method of the text. Each point represents a graphic determination based on at least two concentrations, of a sample prepared from between 70 and 140 embryos, for early points, between 2 and 10 embryos, after stage 18. All points differ significantly from background.

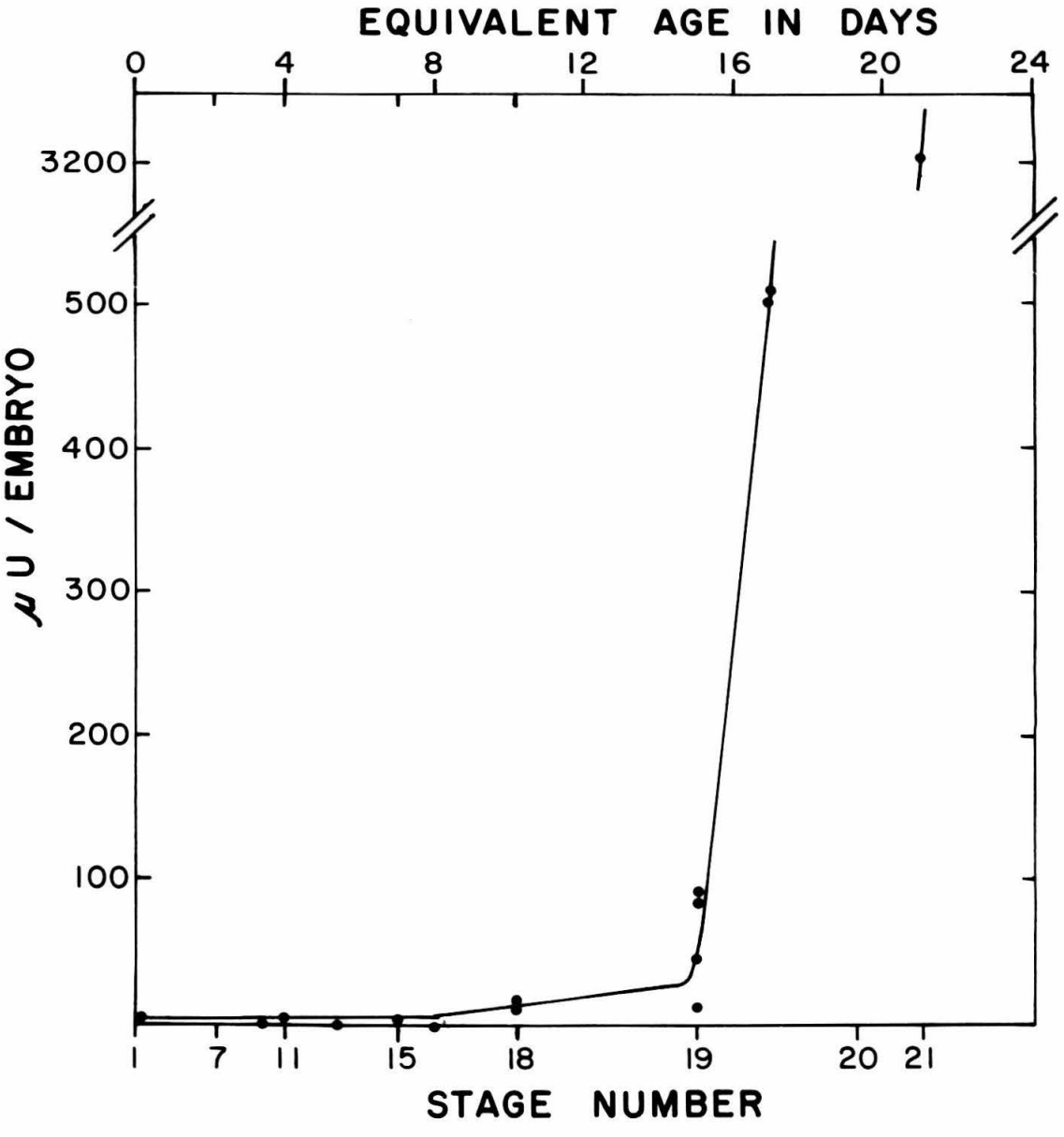


Fig. 11. Amount of xanthine dehydrogenase in normal Rana catesbeiana embryos during development to stage 21, assayed by the radioisotopic method of the text. The samples were the same as those of fig. 10. Each point is based on at least two assays. Determinations at stages 10, 12, and 16 gave indications of activity which were not proven significantly different from background.

background by amounts which are not statistically significant (at the 67 per cent confidence level).

On both the uricase and xanthine dehydrogenase developmental plots, a smooth curve has been fitted to the early points, although they fluctuate considerably, since the scatter seems to be random. The variations may describe true fleeting ontogenetic changes, but much more closely spaced samples would be required to detect them.

2. Specific Enzyme Activities of Organs

The specific activities of uricase and xanthine dehydrogenase in several representative organs at several developmental stages are shown in figures 12 and 13, respectively, and in table 1. These values are intended as rough estimates, most points representing a single determination, so that all assays could be performed together (in only 16 flasks) within a very short time of sacrificing the animals. The magnitude of the differences, however, justifies the crudeness of the sampling. Two classes, enzyme-rich and enzyme-poor organs, are distinct, with activities differing from one to three orders of magnitude. Fluctuations in one organ's activity along the time axis may or may not represent real changes in an ontogenetic pattern, considering that later points represent single individuals (cf. (123)). The few points which were duplicated,

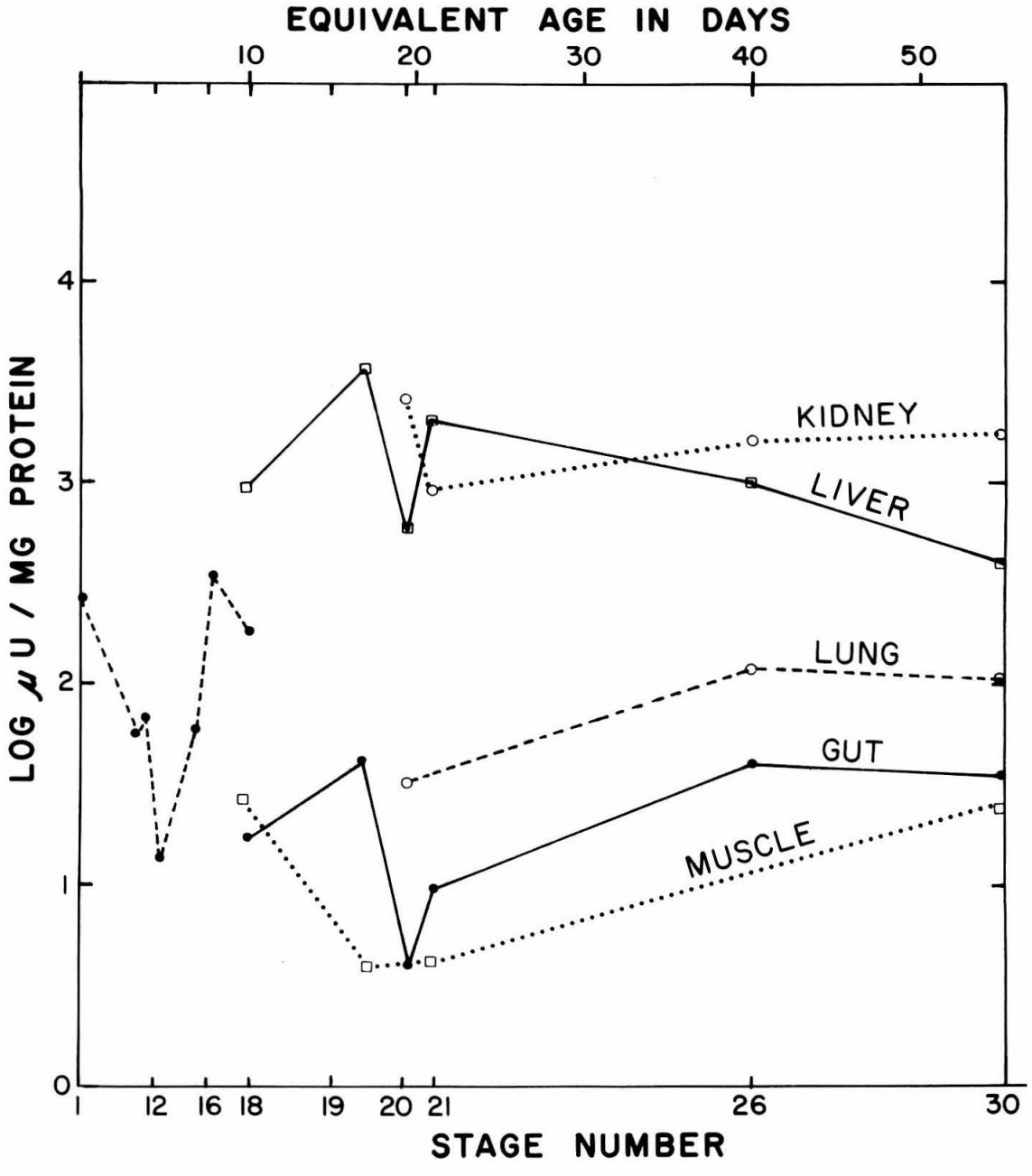


Fig. 12. Specific activity of uricase in whole embryos of early stages and in representative organs of later stages of Rana catesbeiana development. Procedures according to the text. The ordinates are plotted on a logarithmic scale.

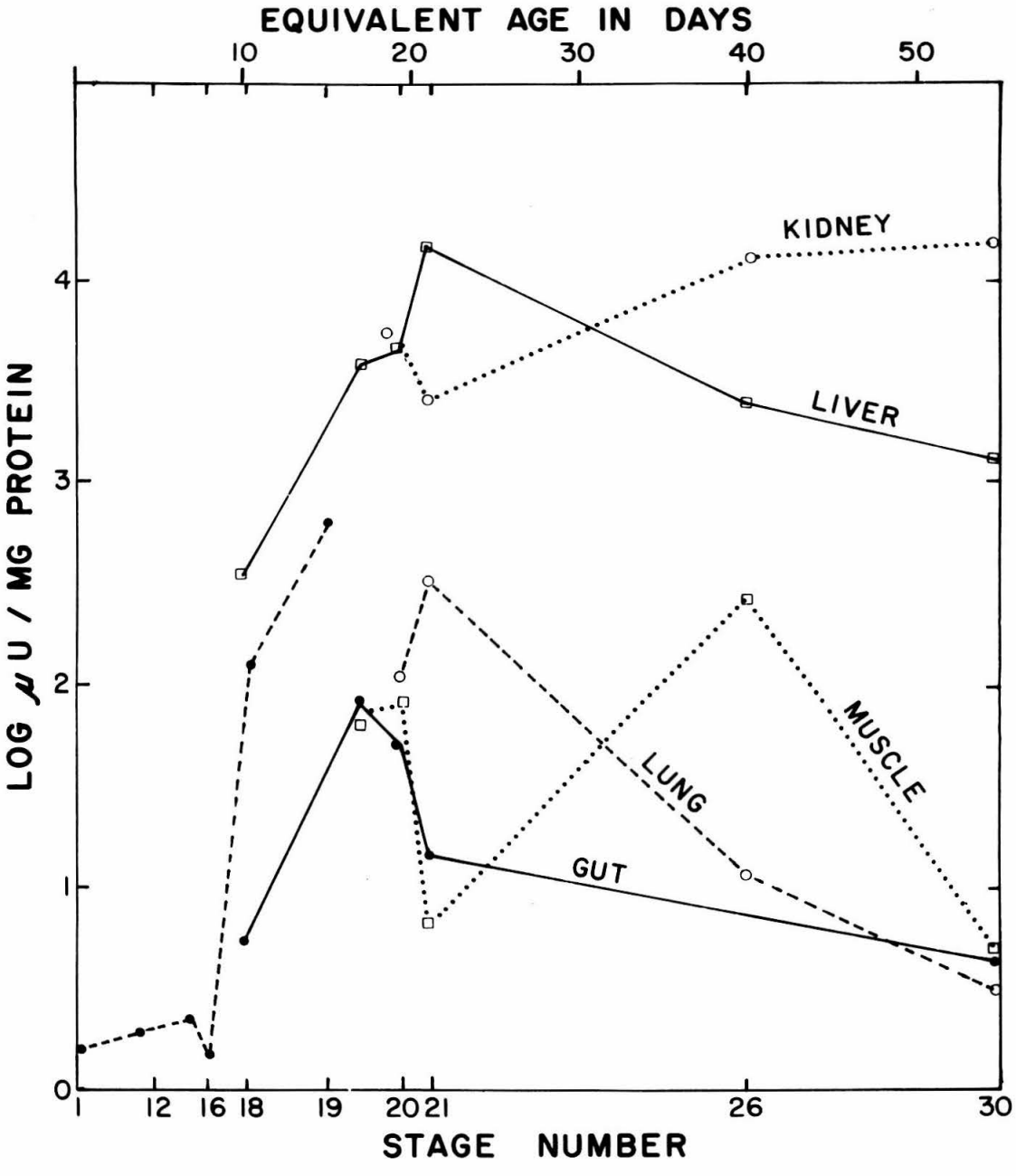


Fig. 13. Specific activity of xanthine dehydrogenase in whole embryos of early stages and in representative organs of later stages of Rana catesbeiana development. Procedures according to the text. The ordinates are plotted on a logarithmic scale.

Table 1. Specific Activities of Adult Rana catesbeiana Organs

Organ	μU Uricase / mg protein ± standard error	μU Xanthine dehydrogenase / mg protein ± standard error
Liver	111. ± 0.8	89.5 ± 0.2
Kidney	487.0 ± 2.7	1040. ± 0.4
Gut	8.53 ± 0.7	0.31 ± 0.12
Skeletal Muscle	6.20 ± 0.4	0.27 ± 0.11
Lung	28.8 ± 0.3	0.08 ± 0.02
		0.34 ± 0.08
Pancreas	6.95 ± 0.3	0.34 ± 0.08
Heart	---	0.68 ± 0.27
Testis	---	0.79 ± 0.14
Ovarian Eggs	2.89 ± 0.07	0.010 ± 0.020
	2.56 ± 0.03	
Blood (whole)	0.14 ± 0.05	0.04 ± 0.011
(serum)		0.16 ± 0.04

in adult specimens, agree rather closely, see table 1.

3. Urate.

Figure 14 represents the few urate determinations which were made. The purine appears to increase fairly continuously through the period sampled. Sudden change at stage 18 is noteworthy by its absence.

D. Discussion

1. Description of Purine Oxidative Enzymes in Ontogeny

Uricase in Rana. When the ontogeny of uricase is compared in catesbeiana (fig. 10) and temporaria (fig. 3), the curves would seem to be about the same within the errors of the methods. The closeness of the units is rather surprising, in view of the appreciably larger size of the temporaria eggs and larvae.

In comparing the two, it is of interest that the activities before stage 18, which the radioisotopic assay is capable of filling in, are actually just at the lower limit of the spectrophotometric method.

Xanthine Dehydrogenase and Uricase. Ontogeny of xanthine dehydrogenase appears to resemble that of uricase quite closely, but the values prior to stage 18 are much lower, and the slope of

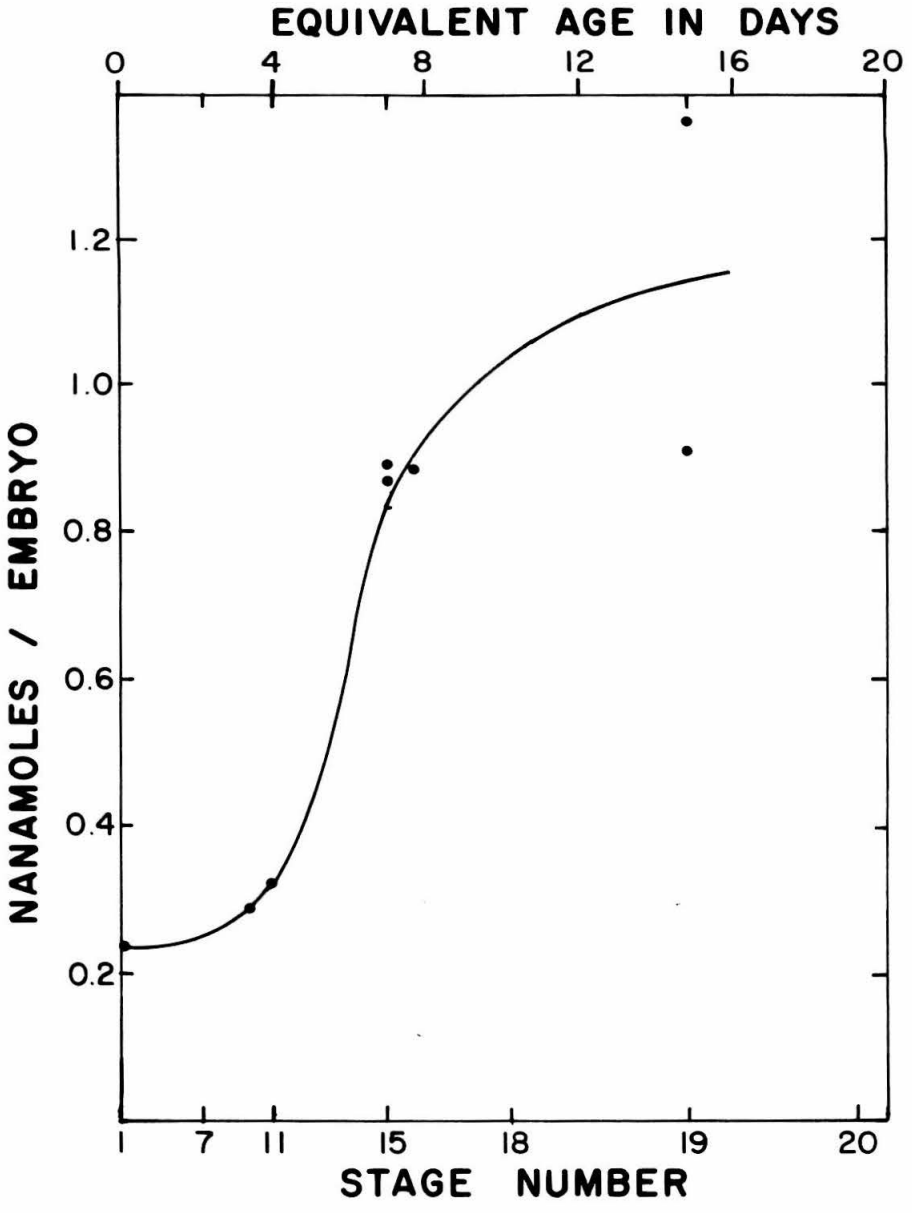


Fig. 14. Amount of urate found in extracts of Rana catesbeiana embryos up to stage 19. The extracts were prepared, gel filtered, and passed through Dowex columns, as detailed in the text, and urate was determined by loss of optical density at 292.5 mμ on uricase treatment.

the accumulation much steeper. Discussion of the similarity is more meaningful at the organ level.

As figures 12 and 13 and table 1 indicate, liver and kidney show like activities of the two enzymes throughout development, as do the enzyme-poor organs, although in the lower-activity organs, xanthine dehydrogenase has fallen by adulthood to an order of magnitude less than uricase. As outlined in the general introduction, however, activity differences need not indicate true concentration differences. The low embryonic values for instance might be attributable to a preponderance of isozymic forms with lower substrate affinities than later-appearing forms (cf. 69, 36)), or to a high enzyme concentration in preenzyme form, or to a bound inactive form. None of these alternatives convincingly explains the low levels in adults, however.

Agreement with Theories of Enzyme Ontogeny. The developmental patterns found for uricase and xanthine dehydrogenase follow all the principles, discussed in the general introduction, for the ontogeny of specialized enzymes. They show sharp specialization both on the time axis and in organ distribution. They clearly demonstrate the principle that functionally associated enzymes show similar patterns in the same organ, but follow different patterns in different organs.

The expected range of patterns varying from organ to organ is perhaps obscured by the crudeness of the sampling, but the

organ patterns studied lend themselves to classification into two distinct and relatively homogeneous groups, enzyme-rich and enzyme-poor. In this light, uricase is seen to resemble chick alkaline phosphatase (72), with an intermediate embryonic enzyme activity which gives way, as the tissues differentiate, to high and low values in the various tissues. Xanthine dehydrogenase, on the other hand, does not show an early intermediate level, but rather rises transiently to a larval maximum and then is lost again (to conventional detection) in all but a few tissues, in a manner somewhat reminiscent of immunoembryological observations (see (114) for references).

Minimal Molecular Concentrations. No attempt was included in the present work to purify xanthine dehydrogenase, but the enzyme seems closely identifiable with the chicken liver enzyme purified by Remy et al. (88), on the basis of its acceptor requirement and its insensitivity to the inhibitor antabuse. In order to calculate a molecular enzymatic activity (turnover number), Remy's molecular weight and Q_{O_2} were adjusted for the applicable conditions of pH, substrate concentration, and temperature, by the use of Remy's data, and an Arrhenius plot for xanthine oxidase (102). The figure thus arrived at differed only slightly from turnover numbers calculated for xanthine oxidase (4, 44). With this molecular

activity, and a cell count for Rana pipiens derived from DNA content per cell (112) and total DNA per embryo (9), the average number of molecules of xanthine dehydrogenase per cell at stage 15 was approximated as follows:

Enzyme activity per embryo at stage 15 = 0.72 μU

Cell count at stage 15 = 2.4×10^6

Molecular activity = 628

$$\frac{7.2 \times 10^{-13} \frac{\text{mole S}}{\text{min. Embryo}} \times 6 \times 10^{23} \frac{\text{molecules}}{\text{mole}}}{2.4 \times 10^6 \frac{\text{cells}}{\text{Embryo}} \times 628 \frac{\text{mole S}}{\text{mole Enz. min}}} = 280 \frac{\text{molecules}}{\text{cell}}$$

2. Physiological Significance of the Enzymes

In Enzyme-Rich Organs. In part I, the data of figure 4 showed that by the thirteenth day, the liver is not the only major source of uricase. Data collected in part II, but not conveniently included in the graphs, corroborate that at their equivalents of 10, 15 and 17 days of temporaria development, catesbeiana larvae too have relatively large extra-hepatic concentrations of uricase, and of xanthine dehydrogenase also. It can be deduced from figure 11 that these activities reside in kidney primordia.

Both excretory rudiments are present at stage 18, when accumulation of both enzymes will be tentatively assumed to begin.

The *pronephros* is in mid-growth (125), and although its duct is blind, and its excretory usefulness is questionable (93), it is richly bathed in blood. The mesonephros, by contrast, which is to function in the adult, has not yet begun to differentiate from the nephric blastema.

Thus in confirmation of the results of part I the liver, from the onset of the animal's digestive activities, perhaps the pronephros, and certainly the mesonephros as soon as it differentiates, can be considered the functional centers of terminal purine oxidation, by virtue of their intensive concentration of both the necessary enzymes. For one adult frog it was calculated that 99.8 per cent of the xanthine dehydrogenase and 98.6 per cent of the uricase were contained in the two enzyme-rich organs.

In Enzyme-Poor Organs. Less obvious is the physiological significance of the trace amounts of both enzymes found in all other organs tested, in the adult at least in rather similar amounts from organ to organ (table 1), and in roughly 10-fold ratio , with less dehydrogenase than uricase. Might the constancy of the amounts indicate that while the central depots account for terminal catabolism of ingested purines, the remaining cells must have trace amounts for their own intracellular metabolic needs?

An explanation for the smaller amount of xanthine dehydrogenase might then be found in the pivotal role which has been assigned

xanthine dehydrogenase (or oxidase) in the control of purine anabolism. The frequently noted correlation between neoplasia and reduced purine catabolism (35) lent support to the hypothesis, recently favored (84), that deletion of catabolic enzymes and subsequent unrestrained nucleic acid synthesis led to cancer. As figure 2 illustrates, xanthine oxidase does catalyze the irreversible reaction which denies purines further access to the pathways which can lead to their re-utilization in nucleic acid or coenzymes. While uricase is a terminal enzyme, without competition, xanthine oxidase commands a branch point, where guanase, nucleoside phosphorylase, and nucleotide pyrophosphorylase may be competing for its substrates. For guanase at least, the free energy of the reaction would allow free reversibility (19). In mammals, experiments support this hypothesis of xanthine oxidase dominance. In rats, dietary hypoxanthine and xanthine were not incorporated into nucleic acids (35), while in isolated rabbit marrow slices, where xanthine oxidase is undetectable, hypoxanthine is a precursor of both adenine and guanine in a 4: 1 ratio , in nucleic acids (1).

Thus the hypothesis seems tenable that this trace amount of enzyme is instrumental in limiting precursor pools for nucleic acid synthesis and cell division. That the lowest values observed are in the rapidly growing embryo would tend to support the notion. First

steps toward testing it would have to include determining the levels of competing enzymes, in both enzyme-rich and enzyme-poor organs. In alternative hypotheses for control of the enzyme, the roles of polyvalent xanthine dehydrogenase as a pteridine oxidase and a nicotinamide-coenzyme reductase might warrant consideration.

3. Implications for the Control of Enzymogenesis

While in the main the present work has remained at a descriptive level, the results provoke at least three lines of thought about the mechanisms of developmental control of enzyme synthesis.

Coordination of Enzymes. The parallelism of the two enzymatic activities studied, in the enzyme-rich, and differently in the enzyme-poor organs, as development progresses, is the most striking feature of the present results. Solomon (104), studying lactic and malic dehydrogenases in developing chick organs found patterns which show similar features. Evidence can be found in his data for a dichotomy of organ types: liver and muscle are relatively rich in enzyme, with distinctive peaks at the fourteenth day of incubation, while heart and brain are relatively poor in both enzymes, and remain fairly constant. Solomon's enzymes may be considered general of function, however, and the difference between the two organ types does not exceed ten-fold.

These correlations may indicate merely that the need for a given enzyme or series of enzymes arises simultaneously at diverse sites, and that at each site separately, synthetic mechanisms respond to the need. But where parallel patterns are disclosed in organs disparate in both location and function, and where, as in purine oxidation, the enzymes concerned perform a service for the whole organism, humoral intercommunication may also be implicated.

The coordinate rise of the urea cycle enzymes in the amphibian liver is known to depend on thyroxin (10), which certainly effects changes synchronously in many other enzymes (5), but the level of thyroxin's primary activity is unresolved. Where hormonal modes of action have been clarified in recent studies, the hormones seem to vary as widely in their action mechanisms as they do in chemical structure. Clever and Karlson, for instance, have shown a direct effect of ecdysone on the insect chromosome, presumably illustrating hormonal action at the gene level, while Yielding and Tomkins (128) have found that steroidal hormones can effect disaggregation of an enzyme itself. Speculation on possible hormonal influences on the embryonic accumulation of purine oxidizing enzymes, then, seemed fruitless, with one exception. If the pre-digestive enzyme changes in the tadpole were all due to the same humoral agent, then that of alkaline phosphatase would

be included (57), and Moog has devoted extensive researches to endocrine regulation of alkaline phosphatase in avian and mammalian embryos (74). In these forms alkaline phosphatase also arises suddenly at a stage just before birth or hatching, so that a genuine homology with the pre-digesting tadpole seemed a possibility.

Moog has shown that the timing of the phosphatase rise, along with a constellation of events which achieve the functional differentiation of the duodenum, is regulated by adrenocortical hormones (74). The chick, rabbit, and guinea pig have well-developed adrenals at the stage in question (75). But whereas Chieffi (16) claims "clear signs of functional activity of the adrenal cortex at very early stages in the tadpoles (stage 22-23)," (i.e. Kopsch's stage 17-18), Witschi's experiments (124) label the cells of the tadpole's mesonephric blastema at stage 19 (Kopsch) still primitive, since the number of them which will become adrenal can still be varied ten-fold by administration of estradiol at this stage.

Chieffi does have evidence for a later accumulation of frog intestinal phosphatase in mid-metamorphosis, after a loss in pre-metamorphosis. This re-accumulation, at least, is susceptible to hydrocortisone acceleration (15).

Thus the rise of purine oxidizing enzymes in the organs studied may possibly reflect a hormonal regulation, to which kidney and liver are differently susceptible from the other organs.

Moog has emphasized, however, that the corticoid reaction is a general one, perhaps operating at the level of permeability, allowing mobilization of nutrients into already open pathways (74). The hormone interacts with the tissue and helps bring about the visible differentiation, but the tissue must first differentiate a responsiveness to the hormone. Hence, resolution of the question whether the adrenal function regulates the pre-digestive rise in enzymes does not promise a final answer to why uricase and xanthine dehydrogenase appear in kidney and liver at the same time at one concentration, and elsewhere at a different concentration level, but may permit the question to be asked with more precision.

Minimal Level of Enzyme Maintenance. It is an open question how far back enzymatic heterogeneity among the areas of the embryo extends, but the existence of respiratory gradients at earliest stages suggests that at least some enzymes are not evenly distributed. The rough value of 300 molecules per cell of xanthine dehydrogenase at stage 15 invites the speculation that if the liver diverticulum and nephric rudiments were to concentrate 99 per cent of the xanthine dehydrogenase within about 10 per cent of the cells (as do the homologous mature organs), then the 90 per cent of other embryonic cells would contain on the order of three molecules of the enzyme each. Rigorous determination of enzyme contents of portions of embryos at the limits of mensurability might hold appreciable theoretic

significance. The binding to ribosomes of a single molecule of enzyme per microbial cell has been demonstrated for β-galactosidase in an inducible non-induced strain of Escherichia coli (21) and for β-glucosidase in a constitutive yeast hybrid (52). Whether this lower limit is general or has any fundamental significance is not yet clear. Too, the figures are at present averages of many cells, but by techniques at the molecular level like the elegant histofluorimetry of Rotman (90), it should be possible to check the distribution, and ascertain whether any cell exists without at least one molecule of the protein product of one of its genes. The latter technique might also be applicable to properly prepared dissociated embryonic cells.

Control by Subcellular Organelles. Gustafson and Hasselberg (43) have implied a form of subcellular control in the explanation that certain sea urchin embryo enzymes all show similar ontogenetic patterns simply because they are mitochondrial, suggesting that the mitochondrion is perhaps synthesized as a unit. The validity of the theory cannot yet be properly evaluated for mitochondria (80).

The results presented here on uricase emphasize that such a mechanism fails to apply to uricase particles, as it apparently fails to apply to lysosomes (81). The data show that uricase ontogeny is quite different from that of catalase (33), which is also contained in uricase particles, while it parallels that of xanthine dehydrogenase,

in liver where the dehydrogenase was demonstrated to be soluble, the uricase particulate.

Uricase particles are not of constant composition (25), and it is implied that this is so because like lysosomes they are collected together into particles after their synthesis as unbound enzymes (81).

4. Urate Contents of Embryos

The data on urate content in embryos are very limited, particularly about stage 18, when the substrate concentration is of greatest interest. Urate concentration is appreciable in the embryo, starting at about 2×10^{-4} M in early cleavage (egg volume = 1.5 µl), so that if substrate has access to the enzyme, as appears likely from the *in vitro* studies previously mentioned, the enzyme should be working near its maximal rate.

Growth from cleavage to stage 19 entails a volume increase of nearly four-fold, mostly water, so that the increase of urate content shown in figure 14 probably results in a nearly constant concentration. On the other hand, between the last two points on the graph, stage 16 and stage 19, uricase activity has increased perhaps ten-fold, so that the substrate is apparently being formed at a higher rate, and a transient accumulation of urate to a level effective for induction may have occurred and gone undetected. Moreover, in order to exclude rigorously the possibility of a simple substrate inductive action *in vivo*, information on whole embryos would have to be augmented by localized urate concentrations in the liver and kidney rudiments.

As far as the data reach, however, they do not contradict the hypothesis that the amphibian larva synthesizes the enzymes of terminal purine catabolism at high levels in anticipation of digestion,

without an inductive signal from their substrates, enabling the metazoan to regulate its internal environment rather than be regulated by it.

ABBREVIATIONS AND TERMINOLOGY

cpm	:	counts per minute
dpm	:	disintegrations per minute
g	:	the acceleration due to gravity, calculated for the average radius
g-min	:	field integral of acceleration in gravities, over time
K	:	10^3, used absolutely, e.g. 90K g-min would describe a centrifugation of 30,000 gravities for 3 min.
K_M	:	the Michaelis-Menten constant
O.D.	:	optical density
ppt	:	precipitate or sediment
supt	:	supernatant
diffusate and retentate	:	describe those fractions which pass and do not pass, respectively, through dialysis membrane (22)
preenzyme	:	enzyme precursor (49), to replace proenzyme and zymogen
FAD	:	flavin-adenine dinucleotide (49)
NAD	:	nicotinamide-adenine dinucleotide (49), DPN
$NADH_2$	:	reduced nicotinamide-adenine dinucleotide (49), DPNH
NADP	:	nicotinamide-adenine dinucleotide phosphate (49), TPN
$NADPH_2$	:	reduced nicotinamide-adenine dinucleotide phosphate, (49), TPNH

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APPENDIX: PURIFICATION OF FROG URICASE

The purification of uricase was undertaken with several objectives in view, in particular the use of purified enzyme to evoke anti-uricase antibodies. It was hoped that the detection of immunologically reactive, though enzymatically inactive, material in early developmental stages would show that the increase at stage 18 proceeds by an activation process; or that the opposite finding would contribute evidence for de novo synthesis. Unfortunately, the test could not be made, for the purified uricase proved non-antigenic when injected into chicken, which appeared to be the animal of choice (2).

A brief presentation of the purification procedure and findings is appended here, however, in support of the claim that uricase from Rana catesbeiana is a very similar protein to that isolated from swine by Mahler et al. (3).

The only appreciable departure in this work from Mahler's methods lay in the choice of starting material. De Duve's "light mitochondrial" fraction (1), the fluffy layer which overlies the true mitochondria after a moderate centrifugation, was used in place of Mahler's "whole mitochondrial" fraction.

The extra-mitochondrial localization of uricase was confirmed for frog liver in experiments like the following, which also demonstrates the disruptive effect of Mahler's blending step:

Protocol. 1. Blend approximately equal weights of freshly dissected frog liver for 30 and 120 sec respectively in 0.88M sucrose, 0.029M in K_2HPO_4, and 10^{-4}M in ethylene diamine tetraacetate, using a Waring Blendor at half speed in the cold. Fractionate the two homogenates separately.

2. Centrifuge each homogenate 5600 g-min (6K rpm X 2 min in the SS-34 head of the Servall Refrigerated Centrifuge), to free it of cell debris and nuclei, and then 270K g-min (12K rpm as above X 25 min) to separate particulate from soluble materials.

3. Resuspend the particulate fraction in buffer, centrifuge at 162K g-min (9K rpm as above X 27 min). Decant supt. Resuspend separately the easily dislodged fluffy layer, and the firm pellet underneath.

Results of photometric assays are given in table 2. It is clear that the enzyme is localized in particles distinct from the mitochondria, and blending is freeing the enzyme from the particles into the supernatant. Thus for purification a gentler homogenization technique was sought to keep the enzyme bound until these particles were separated from others. De Duve's single jeopardy method was used (1). The tissue is homogenized with a single high speed stroke of the Potter-Elvehjem grinder (4). After brief centrifugation the supernatant is reserved, and only the remaining tissue fragments and unbroken cells are subjected to rehomogenization.

Table 2: Crude Subcellular Fractionation of Frog Liver Uricase Activity.

Fraction	Preparation	Total mU Uricase	
		Blended 30 sec	Blended 120 sec
Soluble	270 g-min supt	130.0	289.
Total Particulate	" " ppt	98.5	49.2
Light Particles	162K g-min fluffy	34.0	21.4
Mitochondria	" " pellet	4.3	--
Leakage	" " supt	5.2	4.3

Acetone particles prepared in 0.88M sucrose in place of 0.25M, by single jeopardy grinding instead of the rugged blending method, were expected to include fewer damaged particles than did Mahler's preparations. Thus it came as a surprise when the first extraction of the acetone particles in phosphate buffer, which according to Mahler removes "essentially no uricase, but between 75 and 80 per cent of the total protein of the powder," brought the uricase into solution. Possible explanations for the anomaly were not explored, but the step was omitted. Apparently the resultant loss in purification compensated for the gain in selecting the active part of the mitochondrial fraction, for the specific activity following the next (0.15 per cent Na_2CO_3) extraction is comparable to Mahler's.

The purification scheme, including the modifications of the acetone particle preparation and extraction, is given in table 3. Subsequent steps follow Mahler's procedure. Results obtained are compared in table 4. Both yield and specific activity are still parallel in the frog and swine preparations after the ammonium sulfate and heat steps, which argues for the similar nature of the two enzymes involved. In three separate runs, per cent recovery agreed within two per cent.

Starting with 24 large adult frogs, the purification was carried out at one-eightieth scale. By step 9 the concentration of protein had to be considerably diluted from the procedure recommended, in order to keep the operation up to a microliter level. Dilution prevented

Table 3. Scheme for Purification of Uricase from Frog Liver (modified from (3))

1. Homogenize frog liver parenchymal tissue with three strokes (single jeopardy, as explained in the text) of a Potter-Elvehjem homogenizer in 0.88M sucrose, 0.29M K_2HPO_4, 10^{-4}M versene, running 1000 rpm in the cold. Take 9K g-min supt (using the SS-34 head of the Servall Refrigerated model for all centrifugations).

2. Centrifuge 470K g-min. Resuspend fluffy layer in 0.44M KCl.

3. Centrifuge 470K g-min. Resuspend fluffy layer in KCl, add to acetone at -15°C. Suspend the acetone particles in fresh acetone, dry in air.

4. Grind acetone particles in 0.15M Na_2CO_3. Freeze and thaw rapidly twice. Take supt.

5. Take 17-43% saturated $(NH_4)_2SO_4$ fraction.

6. Suspend ppt in water. Heat to 60°C, 5 min. Take low-speed supt.

7. Add $Ca_3(PO_4)_2$ gel, take low-speed supt.

8. Add dry $(NH_4)_2SO_4$ to 21% saturation. Extract ppt three times with 1% Na_2CO_3.

9. Dialyze extract against 0.02M $KHCO_3$. Assay supt. Assay Na_2CO_3 extract from ppt.

10. Pool supt and ppt extract from step 10. Repeat step 10.

Table 3 (continued)

Steps not performed:

11. Mix extract with t-BuOH. Take supt.

12. Dialyze against Tris-versenate. Extract ppt.

13. Take alkaline $(NH_4)_2SO_4$ fractions about 35% saturation.

Table 4. Purification of Typical Frog and Swine Uricase Preparations Compared.

Purification Stage	Frog Liver Preparation				Swine Liver Preparation (3)		
	spac mU*/mg	conc mU/ml	total activity mU	% recovery	spac mU/mg	conc mU/ml	% recovery
1. Crude supt	1.9	21.9	22,600				
4. Carbonate extract	24.6	190.	2,600	100	29.4	268.	100
6. Heat supt	118.	370.	1,670	64	133.	505.	63
7. $Ca_3(PO_4)_2$ gel supt	183.	221.	1,130	43			
8. AlK Am_2SO_4 ppt	-	515.	~ 930	36	759.	5,680	60
9. $KHCO_3$ supt	-	2,500.	950				
ppt	-	518	184				
10. 2nd $KHCO_3$ supt	2,410	1,840	721				
ppt	4,300	1,280	229	9	2,070	40,100	49
11. t-BuOH supt					3,290		43
12. Tris-versenate ppt					8,310		34
13. Alkaline Am_2SO_4					17,300		21

*One unit is defined as the amount of enzyme which catalyzes the transformation of 1 μmole of substrate per minute. Each value has been normalized from the experimental to optimal substrate concentration by the relation $\frac{V}{v} = \frac{(1 + K_M)}{[s]}$. Correction has not been made for temperature differences (22°C in Mahler's work, 30° here) or pH (8.0 in Mahler's, 9.3 in this work).

much of the enzyme from precipitating in step 9. A degree of concentration was effected by drawing water out of the dialysis sac with dry sugar, but the bicarbonate step was still ineffective on repetition, so the small precipitate was extracted, and further operations were abandoned. This extract had 25 per cent of the specific activity of Mahler's purest, ultracentrifugally and electrophoretically pure, preparation.

A spectrum was taken of the supernatant, which had only 2.4 U/mg, for comparison with Mahler's published spectra of porcine preparations with 8.3 and 17.3 U/mg. The spectral characteristics are compared in table 5. The only significant difference, neglecting sharpness of peaks, is the trough at 253 mμ in the frog preparation and at 261 mμ in the pure protein, which may be attributed to nucleic acid contaminants which Mahler has observed to be difficult to remove. The low 280: 330 ratio is considered by Mahler the best diagnostic of copper proteins, contrasting with values for typical, non-metalloenzymes like 17.6 for bovine plasma albumin and 27.5 for globulin.

Several separations by column chromatography were attempted on carbonate extracts at stage 8 of the purification, with little success. The enzyme washed through ECTEOLA and carboxymethylcellulose columns. It adsorbed to DEAE-cellulose, hydroxyl form, and was slowly and very unsharply eluted by a gradient of ammonium carbonate at about 0.15 to 0.2M. Only about one-fifth of the enzymatic activity

Table 5. Spectral Characteristics of Purified Frog and Pure Swine Uricases.

	Frog Prep	Swine Prep (3)	
	2.4 U/mg	17.3 U/mg	8.3 U/mg
λ_{min}	253 mμ	261 mμ	261 mμ
λ_{max}	275 "	276 "	277 "
Deflection point	284 "	286 "	--
Shoulder	291 "	290 "	--
Ratio $\frac{OD_{280}}{OD_{330}}$	7.9	5.55	9.5

could be recovered in fractions with as much as three-fold higher specific activity, so that the method offered no advantage as a substitute for step 9.

In two trials the gradient elution of uricase suggested at least two peaks. Although the peaks were not sharp enough for confident conclusions, any attempt to show heterogeneity of the enzyme might well start with DEAE-cellulose fractionation.

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