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SYNTHESIS AND DEGRADATION OF XANTHINE DEHYDROGENASE IN CHICK LIVER

IN VIVO AND IN VITRO STUDIES

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Summary

The present study describes the (xanthine:NAD^+ oxidoreductase, EC 1.2.1.37) synthesis and degradation of chick liver xanthine dehydrogenase in vivo and in organ cultures. The results indicate that control of xanthine dehydrogenase activity is mediated by changes in the rate of enzyme synthesis, but that degradation rates are unaffected. The results also suggest that xanthine dehydrogenase synthesis occurs through a previously unreported intermediate. Detected in cultures of liver tissue, this intermediate apparently is not converted into an active enzyme. A model of synthesis and degradation for xanthine dehydrogenase proposes that the synthesis of the enzyme is proportional to messenger RNA and includes an inactive enzyme precursor and a second inactive intermediate prior to degradation.

Integrated mathematical solutions describing the concentration of intermediates as a function of time can be found explicitly for simple models. The appendix to this paper extrapolates solutions for one-, two- and three-step models to generate a mathematical solution for an 'n'-step model containing 'n' intermediates. The rate constants in the solutions can be found experimentally.

Introduction

Dietary protein increases the hepatic activity of enzymes involved in nitrogen metabolism and excretion. This phenomenon has been well documented in

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both birds [1—13] and mammals [14—17], although the mechanism by which the enzyme activities are increased is unknown. In birds, which excrete uric acid, the affected enzymes involved in nitrogen elimination are in the purine biosynthetic pathway.

Four enzymes have been investigated extensively in the chick: (1) xanthine dehydrogenase (xanthine:NAD^+ oxidoreductase, EC 1.2.1.37), which converts hypoxanthine to xanthine and then to uric acid; (2) purine-nucleoside phosphorylase (EC 2.4.2.1) which converts inosine to hypoxanthine; (3) tyrosine aminotransferase (EC 2.6.1.5) which transaminates tyrosine and α-ketoglutarate to *p*-hydroxyphenylpyruvate and glutamate; (4) tryptophan pyrrolase (EC 1.13.11.11) which oxidizes tryptophan to *N*-formyl-L-kynurenine. All four enzymes appear to be regulated coordinately by a common mechanism as part of a nitrogen catabolic enzyme system. Typically, xanthine dehydrogenase shows a 10-fold increase, while purine-nucleoside phosphorylase, tyrosine aminotransferase and tryptophan pyrrolase show a 2—4-fold increase [13].

In addition to dietary protein several other agents or conditions have been shown to influence levels of these enzymes: dietary amino acids [13,18], starvation [18—21], insulin [20—22], carbonyl reagents which are vitamin B-6 antagonists, such as hydrazine and amino oxyacetate [11,13,23], 6-aminonicotinamide (which is an NAD antagonist), reserpine (which depletes catecholamines and serotonin) and monoamine oxidase inhibitors (Ref. 20 and Woodward, W.D. and Fisher, J.R., unpublished data), deoxypyridoxine [13] and melatonin [24]. Although these agents affect widely different systems, including direct metabolic, neural and hormonal effects, all are thought to act through a common element. Thus far, the actual mechanism or common element of action has not been determined.

Xanthine dehydrogenase has been studied in more detail than the other enzymes. It requires iron, FAD and molybdenum in the ratio 8 : 2 : 2 and a cyanolyzable persulfide group for activity [19,25]. It differs from the mammalian enzyme xanthine oxidase in that the chick enzyme utilizes NAD as the electron acceptor rather than molecular oxygen. Xanthanine oxidase has been found to have a molecular weight of 300 000 with two subunits between 135 000 and 150 000 [26,27]. Xanthine dehydrogenase also has a molecular weight of 300 000 and two subunits of 130 000—150 000 in both *Drosophila* [28] and chick liver (Nickels, J.S. and Fisher, J.R., unpublished data). By precipitating radioactively labeled xanthine dehydrogenase with rabbit antiserum, it has been found that the increase in activity caused by protein, insulin, amino acids, reserpine and hydrazine is due to increased synthesis of the enzyme [18,22,29]. Hence, xanthine dehydrogenase is induced by these agents. The rate of degradation is unchanged by levels of dietary protein, and yields a half-response time (half-time) of 23 h for the degradation of labeled protein [18].

This report describes studies undertaken to determine the processes involved in the synthesis and degradation of xanthine dehydrogenase both in vivo and in vitro. Placing liver slices in culture perturbs the synthetic processes, but allows for the detection of a previously undetected intermediate. A model based on these results is proposed, along with integrated rate equations which describe the system. Using these equations, rate constants for the processes involved in

this model are determined. A preliminary report of these results has been presented [30].

Materials and Methods

Materials. Babcock B-300 white leghorn cockerels were obtained from the Gulf Coast Hatchery in Quincy, FL, less than 24 h after hatching. Chicks were maintained in heated brooders in continuous lighting with free access to water and Purina Laboratory Chick S-G Chow until used in experiment. Synthetic diets containing various levels of protein were described by Whitney et al. [31]. Actinomycin D, azaserine, bovine anterior pituitary extract were obtained from Calbiochem; McCoy's 5a Medium (modified) from Grand Island Biological Company; (U-^{14}C)-labeled amino acid mixture, [U-^{14}C]glycine from ICN; fetal calf serum from International Scientific Industries; 2-amino-4-hydroxypteridine from K and K Laboratories; (U-^{14}C)-labeled amino acid mixture, [U-^{14}C]glycine from Schwarz/Mann; Hank's balanced salt solution from Microbiological Associates; McCoy's 5a Medium (modified), penicillin/streptomycin solution, calf serum, horse serum from North American Biologicals; casein, corn starch, alphacel, Hegsted salts, lard, vitamin mixture from Nutritional Biochemicals; α-ketoglutarate, inosine, tyrosine, pyridoxal phosphate, sodium diethyldithiocarbamate, NAD, FAD, cycloheximide, puromycin, glycine, insulin, hydrocortisone, thyroxine, melatonin, glucagon, cyclic AMP, hydrazine, iproniazid, dopamine, pyridoxamine, theophylline, pyrogallol from Sigma.

Enzyme assays. Xanthine dehydrogenase enzyme activity was measured by a modification [32] of the procedure reported by Glassman [33]. Specific activities are reported in pmol of substrate oxidized/min per mg wet tissue.

Tyrosine aminotransferase enzyme activity was measured by the method of Diamondstone [34] as modified by Granner and Tomkins [35]. Specific activities are reported in units of 100 pmol of *p*-hydroxyphenylpyruvate formed/min per mg wet tissue.

Radioactive labeling of experiments. Incorporation of radioactively labeled amino acids into xanthine dehydrogenase was measured using immunoprecipitation techniques described previously [18,36]. Cultures were labeled by adding 5 μCi (U-^{14}C)-labeled amino acid mixture in 0.6% NaCl (pH 7.0)/3 ml culture fluid for measurement of xanthine dehydrogenase synthesis and 5 μCi [U-^{14}C]glycine in 0.6% NaCl (pH 7.0)/3 ml culture fluid for measurement of xanthine dehydrogenase degradation. All cultures were pulsed for 3 h unless otherwise noted. Chicks were labeled by injecting intraperitoneally 50 μCi (U-^{14}C)-labeled amino acid mixture or 30 μCi [U-^{14}C]glycine and fed during the labeling period.

Preparation of organ cultures. Chicks were rinsed with 70% ethanol after decapitation. Livers were removed in a sterile transfer hood (BioQuest Sterility Test Cabinet) and placed in McCoy's 5a Medium, modified [37—40], containing 20% fetal calf serum, 20 ml/l antibiotic mixture (10 000 units penicillin/ml and 10 000 μg streptomycin/ml). 5 mg/l FAD, 10 mg/l molybdic acid, and 15 mg/l $FeSO_4$. This medium formulation will be designated as McCoy's Complete Medium. Liver slices (0.2—0.5 mm) were made with a Stadie-Riggs tissue slicer. Slices were placed on stainless steel mesh grids which positioned

the liver slices at the interface of the medium and the air. Two grids were placed in Falcon plastic tissue culture dishes (35 × 10 mm) which contained 3 ml medium. Livers from three chicks were cultured and samples from three cultures were pooled for each data point (one culture from each chick). Culture dishes were placed in specially designed plexiglas gassing chambers in a Napco CO_2 incubator at 38° C. The chambers were flushed with 95% O_2—5% CO_2 and maintained with a constant flow of 180 ml/min with this gas mixture. Cultures with radioactively labeled amino acids were washed in Hank's balanced salt solution or McCoy's Complete Medium without label before assaying.

Results

Specificity of antiserum

In addition to the experiments previously reported to demonstrate antiserum specificity to the in vivo enzyme [18,36], the labeled enzyme was shown to be specifically precipitated by xanthine dehydrogenase antiserum as determined by SDS gel electrophoresis. The xanthine dehydrogenase-antibody precipitate from an in vivo liver sample was boiled with sodium dodecylbenzenesulfonate, SDS, and the protein bands were separated by SDS gel electrophoresis. When the gels were lightly loaded with protein, two closely aligned bands corresponding to the xanthine dehydrogenase subunits and two bands corresponding to the heavy and light antibody peptide chains were found after the gels were stained for protein. When gels were heavily loaded with protein to increase the number of radioactive counts, the two xanthine dehydrogenase bands appeared as a broad single band. In such a preparation, radioactivity was found predominately in the xanthine dehydrogenase-staining band (Fig. 1).

When diffused against antiserum on an Ouchterlony double-diffusion plate, the in vitro liver sample gave a continuous, single immunoprecipitin line with an in vivo sample and with purified xanthine dehydrogenase. Casein antiserum alone did not react with the in vitro sample, and a precipitate formed by the addition of casein and casein antiserum gave only 15% of the total counts in a xanthine dehydrogenase antiserum precipitate. Counts precipitated by control rabbit serum were usually 10% of the total xanthine dehydrogenase counts and were routinely subtracted as background. The labeled enzyme obtained from an in vitro liver sample was also shown to be specifically precipitated by the antiserum as determined by SDS gel electrophoresis.

Synthesis and degradation in vivo

After chicks fed a synthetic diet containing either 25% protein, 50% protein, or 84% protein were switched to a synthetic diet containing no protein, xanthine dehydrogenase enzymatic activity declined to a new steady-state level with a half-time of 16 h (Fig. 2A). (The half-times given in this paper are the times for one-half of the response to occur; not necessarily equal to an exponential half-life as the kinetics may be different.) The rate of loss of enzyme activity was found to be independent of dietary protein level. This is comparable to results [18] when chicks were starved after maintenance on diets of varied protein content.

The rate of synthesis of immunoreactive xanthine dehydrogenase showed a

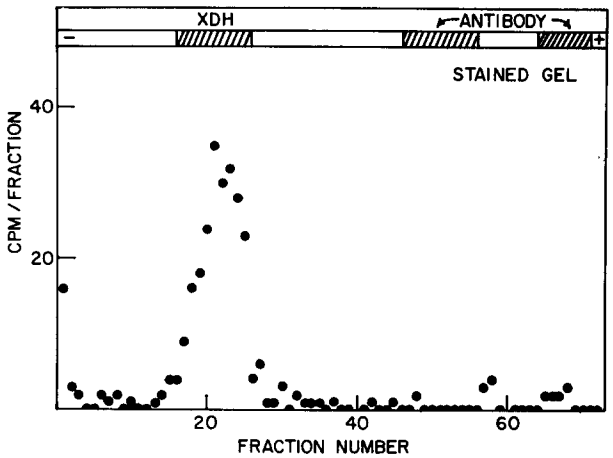


Fig. 1. Labeled amino acid incorporation into xanthine dehydrogenase subunits. A liver preparation was labeled in vivo in chicks fed a 25% protein diet. The enzyme was precipitated with xanthine dehydrogenase antiserum and then treated with 2% SDS. The protease inhibitor, phenylmethyl sulfonyl fluoride, was added to eliminate proteolysis. The proteins were separated by electrophoresis first on a 2.5% acrylamide stacking gel with 0.1% SDS, then on a 7.5% acrylamide gel with 0.1% SDS. The presence of protein was determined by Coomassie blue stain. 1 mm slices were dissolved in H_2O_2 and counted for radioactivity with a Packard scintillation counter in Omnifluor scintillation fluid. The upper diagram shows the location of the xanthine dehydrogenase and antibody proteins. Migration was from left (—) to right (+). Only a single band is seen in the xanthine dehydrogenase region. When less total protein is added to the gels, two bands of xanthine dehydrogenase protein are seen. The lower figure shows the location of the incorporated radioactive amino acids. The first few points represent protein trapped in the stacking gel. Values are reported as cpm/fraction. Background counts have been subtracted.

decrease in the ratio of radioactively labeled amino acids incorporated into xanthine dehydrogenase versus total soluble protein when changing from a diet containing 50% protein to one of no protein. Fig. 2B shows a half-time of 8 h for the decline in the rate of xanthine dehydrogenase synthesis. In this experiment, enzyme activity declined with a half-time of 16 h. Fisher et al. [18] have

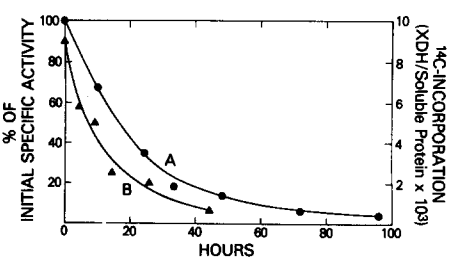


Fig. 2. (A) Xanthine dehydrogenase (XDH) specific activity in chicks switched from a high-protein to a low-protein diet (●). The values are mean averages from 13 experiments in which chicks were switched from a 25% protein to a 0% protein diet, 50% protein to 0% protein, 84% protein to 0% protein, or 50% protein to 5% protein. All diets gave the same half-time when analyzed separately. (B) Rate of xanthine dehydrogenase synthesis in chicks switched from a high-protein to a low-protein diet (▲). Chicks were switched from a 50% protein to a 0% protein diet. The rate of (U-^{14}C)-labeled amino acid incorporation into xanthine dehydrogenase during 3-h pulses was measured at various times. Values are reported as ratios of ^{14}C incorporation in xanthine dehydrogenase to ^{14}C incorporation in total soluble protein and are an average of four experiments.

reported that there is no difference in the rate of degradation between chicks fed a 50% protein or a 0% protein diet. No lag period was observed in the loss of counts in total soluble pool or total soluble protein. When degradation of immunoreactive dehydrogenase was measured by this method in chicks during a transition from a 50% protein diet to a 0% protein diet, similar results were found.

Synthesis and degradation in vitro

The experiments reported here describe an organ culture system of neonatal chick liver. Cultures of embryonic chick liver were not suitable, even though the techniques have been thoroughly described, because embryonic chick liver does not have xanthine dehydrogenase activity [41—44]. The activity of this enzyme increases only after hatching. Cell cultures of neonatal liver were unsuccessful due to their poor viability, overgrowth by undifferentiated fibroblast cells, and poor yield of the time-consuming technique required to separate the cells. Organ cultures did not have these flaws. Thus, it should be possible to test the inducing agents directly on the liver in culture and measure changes in the activity, synthesis and degradation of xanthine dehydrogenase in response to these substances.

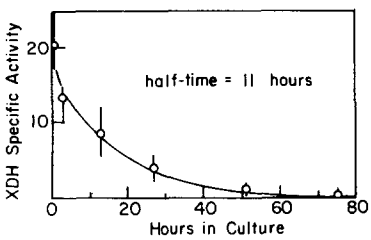
Liver slices from chicks fed a 25% protein synthetic diet were cultured on the air-medium interface with McCoy's Complete Medium at 38°C with 95% O_2—5% CO_2. During the first three days in culture xanthine dehydrogenase enzymatic activity of the liver declined to zero with a half-time of 11 h (Fig. 3). A similar decline was seen in xanthine dehydrogenase activity when chicks were fed either a 50% protein or a 0% protein diet.

Culture medium may not contain those factors normally found in the animal necessary for activation or stabilization of the enzyme. In an attempt to discover this factor, many agents were added to the liver in culture. These agents included four different sera, including chick serum; two media; increased levels of xanthine dehydrogenase cofactors; high levels of amino acids; hormones; and vitamin B-6 antagonists. The latter three agents induce xanthine dehydrogenase in vivo. None of these substances prevented the loss of enzyme activity.

To test for the presence of an inhibitor to xanthine dehydrogenase activity in culture, liver homogenates from culture and from intact chicks were mixed. No decrease was seen in enzyme activity from intact chicks which reduces the likelihood that an inhibitor could account for the decline of activity in culture.

Cell death was not responsible for the decline in enzymatic activity. Synthesis of total soluble protein increased for at least 48 h, and was sensitive to amino acid availability, oxygen tension, protein synthesis inhibitors and an RNA synthesis inhibitor, actinomycin D. In addition, the enzyme tyrosine aminotransferase remained at a constant level in culture.

The ability to synthesize xanthine dehydrogenase relative to soluble proteins as measured immunochemically continues at a constant level for at least 48 h (Fig. 4), even though enzyme activity declines during the same time. Newly synthesized total soluble protein and immunoreactive xanthine dehydrogenase increased during the course of the experiment, but at a constant ratio. The level of synthetic capability was dependent upon the diet of the donor chick,



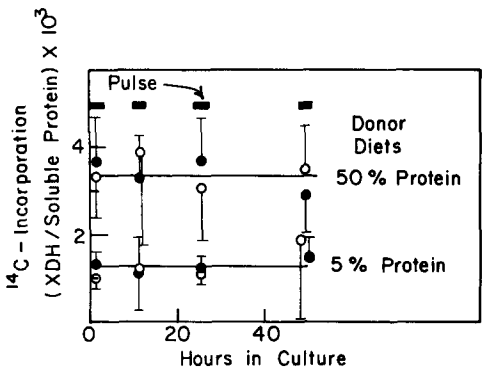


Fig. 3. Xanthine dehydrogenase (XDH) specific activity as a function of time in culture. Liver slices from chicks fed a 25% protein synthetic diet were cultured in McCoy's Complete Medium at 38°C with 95% O_2—5% CO_2. Livers from three chicks were cultured and samples from each chick were pooled for each assay. Bars are ranges of values in four experiments.

Fig. 4. The rate of label incorporation into xanthine dehydrogenase (XDH) relative to soluble protein during 3-h radioactive pulses over time in culture. Liver slices from three chicks fed either a 50% protein (upper line) or a 5% protein (lower line) diet were cultured in McCoy's Complete Medium (•) or Hank's balanced salt solution (○). The averages of six experiments for 50% protein fed donors and three experiments for 5% protein fed donors are presented. Data (not shown) from experiments in which 25% protein fed donors were used also showed a constant level of xanthine dehydrogenase synthesis. Nonspecific precipitated counts were subtracted from the data.

but could not be perturbed by culture conditions (e.g. addition or deletion of amino acids, addition of hormones, or the use of serum from chicks with high xanthine dehydrogenase levels). The synthetic capability for immunoreactive xanthine dehydrogenase responded to oxygen tension, protein synthesis inhibitors, and an RNA synthesis inhibitor (Fig. 5). As with soluble protein, when actinomycin D is withdrawn, immunoreactive dehydrogenase synthesis increased.

The degradation of immunoreactive xanthine dehydrogenase in culture was studied by labeling the enzyme with [U-^{14}C]glycine both in vivo and in vitro. At various times after injecting chicks with 30 μCi [U-^{14}C]glycine, livers were cultured in McCoy's Complete Medium plus 2 mg/ml unlabeled glycine, and the loss of radioactivity over time was measured. It was discovered that if livers were cultured 3 h after the pulse of labeled glycine, only 50% of the labeled enzyme was degraded; a pool of labeled enzyme remained which did not degrade (Fig. 6). Under the same conditions, the labeled enzyme was completely degraded in vivo [18]. If cultured 4 h after the pulse, a smaller pool of non-degradable immunoreactive xanthine dehydrogenase was found. If cultured 6 h after the pulse, the enzyme was completely degraded in culture. Apparently between 3 and 6 h after the pulse in vivo, the non-degradable pool of enzyme is converted into a degradable form. The initial decline of labeled enzyme with a half-time of 30 h is comparable to the in vivo degradation half-time [18]. The experiment using liver samples 3 h after a radioactive pulse was repeated nine times; all showed a non-degradable pool of labeled enzyme. The average of three experiments is shown. Soluble protein and total soluble counts showed

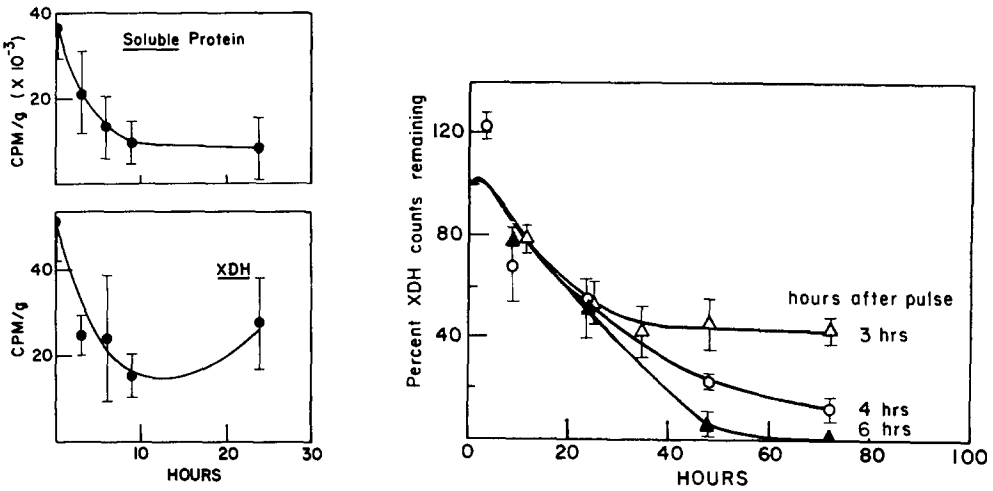


Fig. 5. The effect of actinomycin D on protein synthesis. Radioactive counts incorporated into soluble protein (upper figure) or xanthine dehydrogenase (XDH lower figure) during 3-h pulses (of 5 μCi (U-^{14}C)-labeled amino acids) over time in culture. At time zero 2 μg/ml of actinomycin D were added. Samples are from three chicks fed a 25% protein diet cultured in McCoy's Complete Medium. The average of three experiments is presented. Nonspecific precipitated counts were subtracted.

Fig. 6. The degradation of counts incorporated into xanthine dehydrogenase (XDH) as a function of time in culture. Chicks fed 50% protein diets were injected with 30 μCi of [U-^{14}C]glycine at various times prior to culture of their livers. Livers were cultured in McCoy's Complete Medium plus 2 μg/ml of unlabeled glycine. The average of three experiments is presented in each curve. Nonspecific precipitated counts were subtracted. Livers cultured from chicks following: △, 3 h after pulse; ○, 4 h after pulse; ▲, 6 h after pulse.

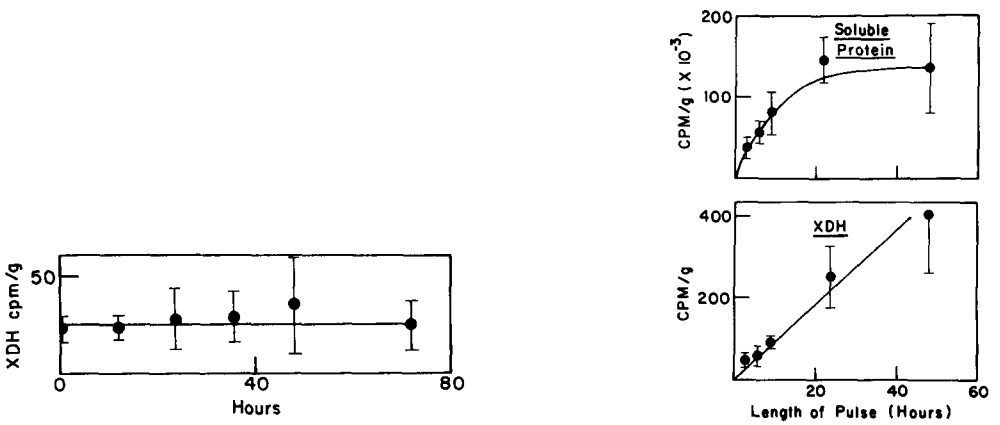


Fig. 7. The degradation of counts incorporated into xanthine dehydrogenase (XDH) as a function of time in culture when liver slices were pulsed in culture for 3 h with 5 μCi of [U-^{14}C]glycine. The slices from three chicks fed a 50% protein diet cultured in McCoy's Complete Medium were washed after the 3 h pulse and fresh medium plus 2 μg/ml of unlabeled glycine was added. The average of three experiments is presented. Nonspecific precipitated counts were subtracted.

Fig. 8. The incorporation of radioactive label into soluble protein (upper curve) and xanthine dehydrogenase (XDH, lower curve) in liver slices in culture continuously exposed to the label. Livers from three chicks fed a 25% protein diet were cultured in McCoy's Complete Medium and 5 μCi of (U-^{14}C)-labeled amino acids were added. The average of three experiments is presented. Nonspecific precipitated counts were subtracted.

a complete decline in counts in all experiments. Enzyme activity decreased as before.

Xanthine dehydrogenase was labeled in vitro by adding 5 μCi [U-^{14}C]glycine to each culture (3 ml culture medium) for 3 h, followed by washing the slices and adding fresh medium plus a chase of unlabeled glycine. It was found that the immunoreactive xanthine dehydrogenase synthesized in vitro was not degraded in culture as no loss of counts was seen by 72 h (Fig. 7). Both soluble protein and total soluble counts showed a decline of counts.

The incorporation of counts into immunoreactive xanthine dehydrogenase in vitro was also measured with a constantly applied source of label. The results in Fig. 8 show that counts incorporated into soluble protein reached a steady-state by 24 h while counts incorporated into immunoreactive xanthine dehydrogenase still increased for at least 48 h.

Discussion

Cells or tissue in culture are often released from normal regulatory control. While some cells may persist in their differentiated function, others may lose it either irreversibly or temporarily, the latter being inducible under the correct conditions [45,46]. Several laboratories have reported the loss or decline of enzyme activity from liver cells in culture. Ornithine transcarbamylase declined to a new steady-state level in mouse liver cultures [47]. Cultures of rat liver have been shown to have a specific activity of 3.7 units for glucose 6-phosphatase compared to 70 units in the donor livers [48]. Another study [49] of rat liver cultures showed a loss of isozymes typical of undifferentiated fetal tissue. Bonney and coworkers [50,51] have also shown that levels of tyrosine aminotransferase activity in rat liver cells decline over 5—6 days in culture. Phenylalanine hydroxylase activity decayed with a half-time of 6 h in rat fetal liver organ culture [52]. The decline and loss of xanthine dehydrogenase enzyme activity in chick liver organ cultures can now be added to this list.

Loss of differentiated characteristics of cells in culture has been attributed to two possible factors. It may be due to an artifact of the culturing method, in which case the trait still could be expressed under the correct conditions [53, 54], e.g., the assumption of a loss of synthesis of phenylalanine hydroxylase which may be caused by the absence of a hepatic or extrahepatic signal [52]. Alternatively, it may be due to an intrinsic cellular change, in which case the presence of an inducer or activator would have no effect [53].

Loss of enzyme activity in vivo when chicks are switched from a high- to a low-protein diet is solely due to a decrease in synthesis of the enzyme (Fig. 2), since the rate of degradation does not change [18]. Because the decline in enzyme activity follows a different time course than the degradation of labeled enzymes, it may be proposed that it is not the active enzyme which is directly degraded, but an inactive intermediate. The degradation time course could reflect reutilization of the labeled amino acid. However, use of a single labeled amino acid which is rapidly excreted (i.e. glycine) reduces this problem as reported by Fisher et al. [18]. Further, no lag is seen in the decline of counts in total soluble protein. Therefore, a two-step process of enzyme inactivation and then degradation best fits the data rather than a simple first-order decline to degradation.

The results in vitro show that the synthesis of immunoreactive xanthine dehydrogenase proceeds at levels predetermined in the chick (Fig. 4); however, enzyme activity decreases (Fig. 3). In culture, chick liver apparently synthesizes an inactive form of xanthine dehydrogenase which is immunochemically similar to active xanthine dehydrogenase found in vivo, but this inactive form cannot be processed to an active enzyme. The active enzyme initially present in the cultures must have been formed and activated in vivo and then degraded in a normal manner. The rate of degradation of this preformed enzyme does not change in culture. Increased rates of degradation cannot account for the complete loss of xanthine dehydrogenase activity. Instead increased degradation would cause only a new steady-state at a lower enzyme concentration. Further, the results of Fig. 7 suggest that the inactive immunoreactive xanthine dehydrogenase synthesized in vitro cannot be degraded. Other properties of xanthine dehydrogenase synthesis in culture appear to be unaffected by removal from the intact chick.

Changes in the rates of enzyme synthesis and degradation can regulate the concentration of a specific enzyme [55,56]. Models describing the synthesis and degradation of intermediates in the formation and destruction of enzymes can be formulated as an aid in understanding the regulation of enzyme concentrations [55,57]. The models do not always describe distinct reactions, but simplify the synthetic or degradative processes to a single zero- or first-order process for each intermediate. The models of interest in this paper allow only the first synthetic process to be regulated (i.e. the rate constant is perturbed).

The following model of synthesis and degradation of xanthine dehydrogenase is consistent with the results obtained thus far:

$$\xrightarrow{k_a} \underset{\downarrow k_1}{M} \xrightarrow{k_b} I_1 \xrightarrow{k_2} E \xrightarrow{k_3} \underset{\downarrow k_4}{I_2}$$

Dietary protein and other agents regulate the synthesis of messenger RNA, M, for xanthine dehydrogenase which, in turn, directly determines the rate of enzyme synthesis and, hence, the concentration of the enzyme. The results suggest that messenger RNA is translated into an inactive xanthine dehydrogenase (I_1) which is then converted into active enzyme, E. Prior to degradation, the active enzyme is converted to a second inactive form (I_2) which may then be degraded. The three enzyme forms, I_1, E and I_2 are all immunochemically similar. Only the mRNA, M, and the inactive enzyme, I_2, can be degraded. The model represents a series of synthetic and degradative processes for each intermediate, but is not meant to imply distinct reactions. Instead synthetic and degradative processes represent the formation and loss, respectively, of substances in an undetermined (or undefined) manner (e.g. k_b is the rate constant representing only the formation of I_1, not the loss of M). The processes experimentally result in a single rate constant, not for a single reaction, but for a combination of reactions, one of which is rate-limiting. The process is undoubtedly more involved. This model is under the following restrictions: (1) all processes are essentially irreversible; (2) $k_1 \neq k_2 \neq k_3 \neq k_4$; (3) only the first step, k_a, which is zero-order, is changed from the initial to final steady-states; (4) all other proccesses are simplified as first-order; (5) changes in the

levels of synthesis of M always start and end at a steady-state.

In organ cultures of chick liver slices, synthesis of xanthine dehydrogenase continues as detected by precipitation of radioactively labeled enzyme with specific antiserum, while specific enzymatic activity declines to zero over the same time period. It is suggested that an inactive, but immunochemically similar, form of the enzyme is synthesized in culture, but the conversion of inactive enzyme, I_1, to active enzyme E, is blocked upon culturing. Thus, active enzyme formed in vivo loses activity in culture and is degraded, while detection of the prelabeled inactive precursor, I_1, continues. The level of enzyme synthesis is reduced by treatment with actinomycin D, hence, synthesis is proportional to mRNA concentration. In culture, the I_1 form which is continually synthesized was found not to be degraded, rather its concentration continually increased. When xanthine dehydrogenase is labeled in vivo and then cultured, the labeled active enzyme, E, is converted to the second inactive enzyme, I_2, and is degraded. Any of the I_1 form of the enzyme labeled in vivo cannot be processed to E in culture, and would be detected as a non-degradable pool. The size of the labeled non-degradable pool decreased as the time after the pulse in vivo increased, since more of the labeled I_1 form was converted to active enzyme, E, in vivo.

That degradation is a two-step process is seen both in vivo and in vitro. The half-time for enzyme degradation is much longer than the half-time for loss of enzyme activity, produced either by culturing or by a change in the level of dietary protein. Hence, the two are likely separate processes. It appears that the enzyme responsible for degradation of xanthine dehydrogenase recognizes only the I_2 form and not I_1or E. Evidence from other systems shows specificity of action of proteases [58,59].

Integrated rate equations can easily found for this model (see Appendix). The following equations describe the system as a function of time:

$$\frac{d(M)}{dt} = k_a - k_1 M$$

$$\frac{d(I_1)}{dt} = k_b M - k_2 I_1$$

$$\frac{d(E)}{dt} = k_2 I_1 - k_3 E$$

$$\frac{d(I_2)}{dt} = k_3 E - k_4 I_2$$

Using the following steady-state conditions:

$k_a = k_1 M_0$, $k'_a = k_1 M_f$

$k_b M_0 = k_2 I_{10}$, $k_b M_f = k_2 I_{1f}$

$k_2 I_{10} = k_3 E_0$, $k_2 I_{1f} = k_3 E_f$

$k_3 E_0 = k_4 I_{20}$, $k_3 E_f = k_4 I_{2f}$

where k_a and k'_a are the different rates of transcription at the initial and final steady-states, respectively, the following integrated solutions result:

$$F_M = e^{-k_1 t}$$

$$F_{I_1} = -\frac{k_1}{k_2 - k_1} e^{-k_2 t} - \frac{k_2}{k_1 - k_2} e^{-k_1 t}$$

$$F_E = \frac{k_1 k_2 e^{-k_3 t}}{(k_3 - k_1)(k_3 - k_2)} + \frac{k_1 k_3 e^{-k_2 t}}{(k_2 - k_1)(k_2 - k_3)} + \frac{k_2 k_3 e^{-k_1 t}}{(k_1 - k_2)(k_1 - k_3)}$$

$$F_{I_2} = -\frac{k_1 k_2 k_3 e^{-k_4 t}}{(k_4 - k_1)(k_4 - k_2)(k_4 - k_3)} - \frac{k_1 k_2 k_4 e^{-k_3 t}}{(k_3 - k_1)(k_3 - k_2)(k_3 - k_4)}$$
$$- \frac{k_1 k_3 k_4 e^{-k_2 t}}{(k_2 - k_1)(k_2 - k_3)(k_2 - k_4)} - \frac{k_2 k_3 k_4 e^{-k_1 t}}{(k_1 - k_2)(k_1 - k_3)(k_1 - k_4)}$$

The term F_X defined in Appendix describes the fraction of the change in the amount of an intermediate which remains to occur at any time in proceeding from one steady-state to another. Most important is that by measuring the concentrations of the intermediates the rate constants can be determined. The solutions presented above describe this model system for the in vivo case in chick liver. In cultures of chick liver, however, the step I_1 to E is blocked. Therefore, the model now becomes:

$$\xrightarrow{k_a} M \xrightarrow{k_b} I_1 \quad \text{and} \quad E \xrightarrow{k_3} I_2$$
$$\downarrow k_1 \qquad\qquad \downarrow k_4$$

Solutions for this model are:

$$F_M = e^{-k_1 t}$$

$$F_E = e^{-k_3 t}$$

$$F_{I_2} = -\frac{k_3}{k_4 - k_3} e^{-k_4 t} - \frac{k_4}{k_3 - k_4} e^{-k_3 t}$$

In chick liver cultures, I_1 is not degraded; therefore, it does not start at nor reach a steady-state and the solution to its rate equation cannot be found by this method. Alternatively, for any concentration of M,

$$I_1 = k_b M t$$

which is found by integrating the rate equation

$$\frac{d(I_1)}{dt} = k_b M$$

for any constant M.

By plotting measured values of ln F_X versus time for each intermediate the rate constants can be found. The ln F_M versus time yields a straight line with slope equal to $-k_1$. By perturbing the steady-state concentration of M with actinomycin D, the data can be plotted to yield a value for the rate constant k_1 of 0.092 h^{-1} with a half-response time of 8 h for the degradation of mRNA as shown in Fig. 9A.

The rate constant k_2 cannot be determined directly from liver cultures since this process is blocked. However, a determination of the half-time of this process can be made. When chicks are pulsed in vivo, the amount of label in I_1 relative to the total labeled enzyme pool decreased with time as the labeled I_1 was converted to labeled E and labeled I_2. The amount of labeled I_1 was found by culturing the livers and determining the non-degradable pool of enzyme, I_1. By plotting the ratio of labeled I_1 to the total labeled enzyme pool (found at time zero in culture) versus time, the half-time for the process I_1 to E was found to be 2.7 h (Fig. 10). An estimate of k_2 can be found using its half-time. From the equation, $k = 0.693/t_{1/2}$, k_2 is equal to 0.257 h^{-1}. It should be noted that k_2 also cannot be determined in vivo because the I_1 form cannot be detected in vivo.

Using F_{I_1} presented above, a graph of the ln F_{I_1} versus time gives a curve which approaches a straight line as time increases. The asymptotic slope is equal to k_1 only if k_1 is less than k_2 in this system. Since k_2 is much larger than k_1, previous studies in vivo would not have been expected to have found evidence for the I_1 intermediate as I_1 to E is not a rate-limiting step.

The rate constant k_3 for the in vitro model was found by plotting the ln F_E versus time as in Fig. 9B. This graph yields a straight line slope equal to $-k_3$. The data gives a value for k_3 of 0.056 h^{-1} with a half-time of 11 h for the conversion of E to I_2.

The ln F_{I_2} for the in vitro model versus time does not give a straight line, but rather a curve. If k_4 is less than k_3, the curve will approach a straight line with a slope of $-k_4$ as time increases. This is an example of a two-step process which

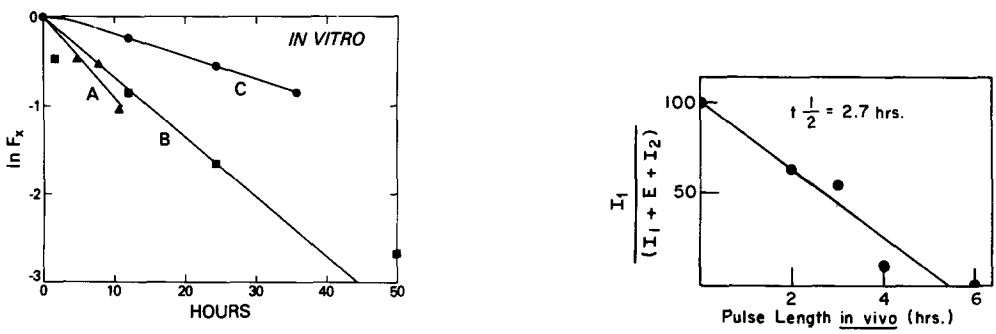


Fig. 9. (A) The ln F_E (E = xanthine dehydrogenase, cpm/g) versus time in culture with 2 μg/ml of actinomycin D added to the culture medium (▲). $E_f = 0$ and $E_0 = 40$. The half-time for the fall in ability to synthesize xanthine dehydrogense is 8 h. The slope of the line is $-k_1$ and is equal to 0.092 h^{-1}. Slope was determined by least squares linear regression method with $r^2 = 0.92$. (B) The ln F_E (E = xanthine dehydrogenase, specific activity) versus time in culture (■). $E_f = 0$ and $E_0 = 21$. The half-time for the loss of xanthine dehydrogenase activity in culture is 11 h. The slope of this line is $-k_3$ and is equal to 0.056 h^{-1}. Slope was determined by least squares linear regression method with $r^2 = 0.96$. (C) The ln F_E (E = xanthine dehydrogenase, cpm/g) versus time in culture for a degradation experiment (●). $E_f = 0$ and $E_0 = 140$. The half-time for degradation of xanthine dehydrogenase in culture is 30 h. The limiting slope of this curve is $-k_4$ and is equal to 0.025 h^{-1}. Slope was determined by least squares linear regression method with $r^2 = 0.99$.

Fig. 10. The fraction of counts incorporated into I_1 relative to the total counts incorporated into I_1 + E + I_2 during the time after an in vivo pulse. I_1 is not degradable in culture and is determined from the data in Fig. 6. This line gives a half-time of 2.7 h for the processs I_1 to E.

gives the appearance of a lag before a straight line decay is approached. The data for the degradation of label from E and I_2 in culture plotted in this way (Fig. 9C) yield a rate constant k_4 of 0.025 h^{-1} with a half-time of 30 h. Since the rate constants for degradation and for loss of activity of xanthine dehydrogenase in culture are not equal, further support is given to the existence of the inactive form, I_2. If the data for degradation had yielded a straight line with a rate constant the same as k_3, it would be necessary to propose a model in which E is directly degraded and loss of activity occurs through degradation rather than through conversion to an inactive form.

Similar rate constants are found for the in vivo model using data collected in vivo in chick liver. When values for the decreasing rate of xanthine dehydrogenase synthesis in chicks switched from a 50% protein to a 0% protein diet are treated by the method presented here, the rate constant k_1 can be found. This decrease in the ability to synthesize xanthine dehydrogenase should be proportional to the concentration of mRNA. The data can thus be treated as measuring M indirectly. A graph of the ln F_M versus time yields the rate constant k_1 of 0.097 h^{-1} with a half-time of 8 h (Fig. 11A). By similarly treating the decline in enzymatic activity, E, during the change in diet from 50% to 0% protein, the rate constant k_3 is found to be 0.066 h^{-1} with a half-time of 16 h (Fig. 11B). Analysis of the data yields a rate constant for degradation, k_4, of 0.024 h^{-1} with a half-time of 23 h (Fig. 11C). Half-times for degradation of 23 h [18] and 30 h [19] have been reported for xanthine dehydrogenase.

Although the culture system does not truly represent in vivo conditions, the

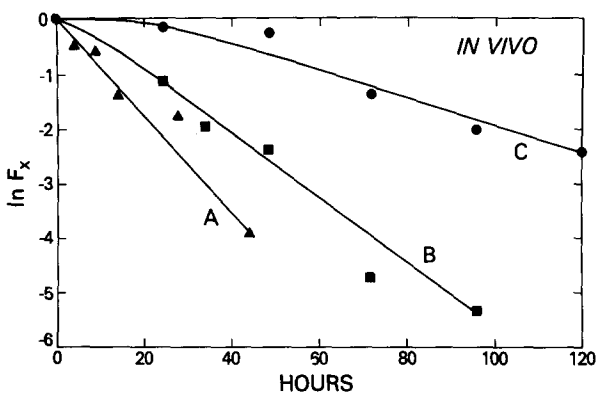


Fig. 11. (A) The ln F_E in vivo (E = xanthine dehydrogenase, cpm/g) versus time (▲). Chicks fed a 50% protein diet were switched to a 0% protein diet and the rate of enzyme synthesis was determined at various times. The half-time for the fall in ability to synthesize xanthine dehydrogenase is 8 h. The slope of the line is $-k_1$ and is equal to 0.097 h^{-1}. Slope was determined by least squares linear regression method with r^2 = 0.93. (B) The ln F_E in vivo (E = xanthine dehydrogenase, specific activity) versus time (■). Chicks fed a 50% protein diet were switched to a 0% protein diet and the decline in enzymatic activity was measured. The half-time for the decline in enzymatic activity is 16 h. The limiting slope of this curve is $-k_3$ and is equal to 0.066 h^{-1}. Slope was determined by least squares linear regression method with r^2 = 0.98. (C) The ln F_E (E = xanthine dehydrogenase, cpm/g) versus time for a degradation experiment in vivo (●). Chicks fed a 50% protein diet were pulsed for 3 h with [U-^{14}C] glycine and the decline in counts incorporated into xanthine dehydrogenase was determined. The half-time for the decline in counts is 23 h. The limiting slope is $-k_4$ and is equal to 0.024 h^{-1}. Slope was determined by least squares linear regression method with r^2 = 0.99. Data are from Fisher et al. [18].

similarity in rate constants for loss of enzyme activity, degradation of enzyme and dependence of enzyme synthesis on mRNA suggests that the same processes occur in the two systems. The novel discovery of an inactive form of the enzyme which is detected only in a culture system warrants further study to detect the inactive form in vivo and its conversion to active enzyme.

Appendix

Models can be developed [55—57] to describe the processes involved in the synthesis and degradation of enzymes. Mathematical solutions have been found for these models which describe the system as a function of time and allow the determination of the degradative rate constants. These early models were either one- or two-step processes. These models simplify the synthetic or degradative processes into a single step for each intermediate which is either zero- or first-order. The rate constant for each step then represents the rate-limiting reaction for that process.

We present here a solution for an n-step model of synthetic and degradative processes which is applicable to enzyme regulation for the case when only the zero-order synthesis rate is perturbed (e.g transcription).

These models are under the following restrictions: (1) all processes are essentially irreversible; (2) $k_1 \neq k_2 \neq k_3 \ldots \neq k_n$; (3) only the first step, k_a, which is zero-order, is changed from the initital to final steady-states; (4) all other processes are simplified as first-order; (5) changes in the levels of the first step should always start and end at a steady-state.

The first and simplest model to be developed assumed a zero-order rate of synthesis and a first-order rate of degradation of active enzyme (or protein). This can be presented in model form as in Fig. A1a. This model was treated by Segal and Kim [56] and led to the equation:

$$\ln(E'_0 - E) = \ln(E'_0 - E_0) - k_2 t$$

MODELS OF ENZYME REGULATION

a. $\xrightarrow{k_1}$ E Segal and Kim, 1963
$\downarrow k_2$

b. $\xrightarrow{c_1}$ N_t $\xrightarrow{c_2}$ M_t Lee, Reel and Kenny, 1970
$\downarrow \lambda_1$ $\downarrow \lambda_2$

c. $\xrightarrow{k_a}$ I_1 $\xrightarrow{k_b}$ I_2 $\xrightarrow{k_c}$ I_3 Fisher and Thompson, 1976
$\downarrow k_1$ $\downarrow k_2$ $\downarrow k_3$

Fig. A1. (a) Single-step model describing zero-order synthesis with rate constant k_1 and first-order degradation with rate constant k_2 of enzyme E. (b) Two-step model describing zero-order synthetic process with rate constant c_1 and first-order degradative process with rate constant λ_1 of material N (e.g. mRNA); and first-order synthetic process with rate constant c_2 and first-order degradative process with rate constant λ_2 for material M (e.g. an enzyme) (from Ref. 60). (c) Three-step model describing synthetic and degradative processes of intermediates I_1, I_2 and I_3. The first step is zero-order; all others are first-order. k_a, k_b and k_c are rate constants for the respective synthetic processes. k_1, k_2 and k_3 are rate constants for the respective degradative processes.

for treated animals, where E'_0 and E_0 are the final and initial concentrations of enzyme respectively, and k_2 is the rate constant for degradation of E. For purposes of this paper we will let $E'_0 = E_f$. Then this equation can be rewritten:

$$\ln\left[\frac{E_f - E}{E_f - E_0}\right] = -k_2 t$$

or

$$\frac{E_f - E}{E_f - E_0} = e^{-k_2 t}$$

The one-step model was expanded to a two-step model [60] as in Fig. A1b. This model gave the equation:

$$M_t = e^{-\lambda_2 t}\left(M_0 + \frac{(N_0 - C_1/\lambda_1)\lambda_2}{\lambda_1 - \lambda_2}[1 - e^{-(\lambda_1 - \lambda_2)t}] - \frac{c_1 c_2}{\lambda_1 \lambda_2}[1 - e^{\lambda_2 t}]\right)$$

where c_1 and c_2 are rate constants for synthesis in the first and second steps respectively, λ_1 and λ_2 are rate constants for degradation in the first and second steps respectively, N_0 and M_0 are concentrations of material at time zero in the first and second steps respectively, and M_t is the amount of material in the second step at any time t. Using the steady-state equations for a change in the rate of synthesis from c_1 to c_1^*

$c_1 = \lambda_1 N_0$ and $c_2 N_0 = \lambda_2 M_0$ at $t = 0$, and

$c_1^* = \lambda_1 N_f$ and $c_2 N_f = \lambda_2 M_f$ at the final steady state,

this equation can be rewritten:

$$\frac{M_f - M_t}{M_f - M_0} = -\frac{\lambda_1 e^{-\lambda_2 t}}{\lambda_2 - \lambda_1} - \frac{\lambda_2 e^{-\lambda_1 t}}{\lambda_1 - \lambda_2}$$

A three-step model presented in Fig. A1c can also be solved. The following equations describe the system as a function of time where loss of a material is only described by the degradative steps (i.e. k_b does not represent a loss of I, but only the formation of I_2):

$$\frac{d(I_1)}{dt} = k_a - k_1 I_1$$

$$\frac{d(I_2)}{dt} = k_b I_1 - k_2 I_2$$

$$\frac{d(I_3)}{dt} = k_c I_2 - k_3 I_3$$

Assuming only k_a changes from k_a to k'_a, $k_1 \neq k_2 \neq k_3$, and using the steady-state conditions for the respective equation

$k_a = k_1 I_{10}$, $k'_a = k_1 I_{1f}$

$k_b I_{10} = k_2 I_{20}$, $k_b I_{1f} = k_2 I_{2f}$

$k_c I_{20} = k_3 I_{30}$, $k_c I_{2f} = k_3 I_{3f}$

the following integrated solutions result:

$$\frac{I_{1f} - I_1}{I_{1f} - I_{10}} = e^{-k_1 t}$$

$$\frac{I_{2f} - I_2}{I_{2f} - I_{20}} = -\frac{k_1}{k_2 - k_1} e^{-k_2 t} - \frac{k_2}{k_1 - k_2} e^{-k_1 t}$$

$$\frac{I_{3f} - I_3}{I_{3f} - I_{30}} = \frac{k_1 k_2 e^{-k_3 t}}{(k_3 - k_1)(k_3 - k_2)} + \frac{k_1 k_3 e^{-k_2 t}}{(k_2 - k_1)(k_2 - k_3)} + \frac{k_2 k_3 e^{-k_1 t}}{(k_1 - k_2)(k_1 - k_3)}$$

These solutions show a pattern that can be extrapolated to 'n' steps for the two models shown in Fig. A2 or any combination of these two kinds of models. In the first model the degradative step for an intermediate is the synthetic step for the following intermediate, whereas in the second model these two processes are distinct. Both models yield the same mathematical solution. The general solution which results is:

$$F_{I_n} = (-1)^{n+1} \sum_{i=1}^{n} A_i^n e^{-k_i t}$$

which is defined in Fig. A3. F_{I_n} is the fraction of change remaining to occur (Fig. A4).

In the special case where $k_1 = k_2 = \ldots k_n$ a general solution can also be found for a three-step model which gives the following functions and steady-state values:

	at steady state:
$\frac{d(A)}{dt} = k_a - k_1 A$	$k_a = k_1 A$
$\frac{d(B)}{dt} = k_b A - k_1 B$	$k_b A = k_1 B$
$\frac{d(C)}{dt} = k_c C - k_1 B$	$k_c C = k_1 B$

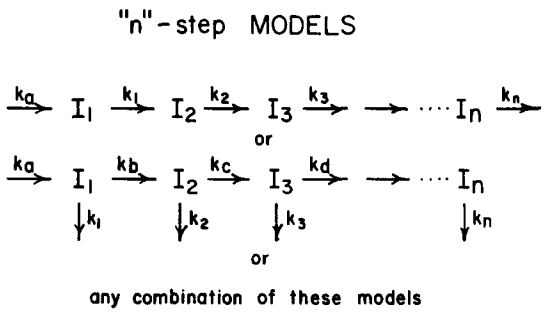


Fig. A2. Models of 'n'-step processes for synthesis and degradation of intermediates $I_1, I_2 \ldots I_n$ with rate constants for synthesis, k_a, k_b . . ., and rate constants for degradation $k_1, k_2 \ldots$; n, number of the intermediates.

A GENERAL MATHEMATICAL SOLUTION

$$F_{I_n} = (-1)^{n+1} \sum_{i=1}^{n} A_i^n \, e^{-k_i t}$$

where N = Number of the intermediate

e.g. N = 1 $F_{I_1} = A_1^1 \, e^{-k_1 t}$

N = 2 $F_{I_2} = -A_1^2 \, e^{-k_1 t} - A_2^2 \, e^{-k_2 t}$

N = 3 $F_{I_3} = A_1^3 \, e^{-k_1 t} + A_2^3 \, e^{-k_2 t} + A_3^3 \, e^{-k_3 t}$

where $$A_i^n = \frac{\pi_{j=1,\, j \neq i}^{n} k_j}{\pi_{j=1,\, j \neq i}^{n} (k_i - k_j)}$$

e.g. n = 1 i = 1 $A_1^1 = 1$

n = 2 i = 1 $A_1^2 = \frac{k_2}{k_1 - k_2}$

i = 2 $A_2^2 = \frac{k_1}{k_2 - k_1}$

n = 3 i = 1 $A_1^3 = \frac{k_2 \, k_3}{(k_1 - k_2)(k_1 - k_3)}$

i = 2 $A_2^3 = \frac{k_1 \, k_3}{(k_2 - k_1)(k_2 - k_3)}$

i = 3 $A_3^3 = \frac{k_1 \, k_2}{(k_3 - k_1)(k_3 - k_2)}$

Fig. A3. A general mathematical solution for 'n'-step models of synthesis and degradation of intermediates $I_1, I_2 \ldots I_n$. F_{I_n} is defined in Fig. A4. Examples are shown for n = 1, 2 and 3.

The following solutions are found:

$$F_A = e^{-k_1 t}$$

$$F_B = (1 + k_1 t)\, e^{-k_1 t}$$

$$F_C = \left(1 + k_1 t + \frac{k_1^2 t^2}{2}\right) e^{-k_1 t}$$

MATHEMATICAL SYMBOLISM

$$F_{I_N} = \frac{[I_N]_F - [I_N]}{[I_N]_F - [I_N]_0}$$

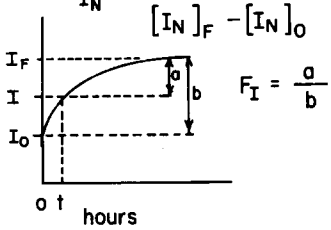


Fig. A4. The definition of F_{I_n} which represents the fraction of the change left to occur in the concentration of I. $I_f - I_0$, total amount of increase or decrease; $I_f - I$, amount of change left to occur.

The pattern to these solutions can be extrapolated to yield the general equation for the case $k_1 = k_2 = \ldots k_n$

$$F_N = e^{-kt}\left(1 + \sum_{i=1}^{n} \frac{k^i t^i}{i}\right)$$

The mathematics involved in these solutions has been presented by several authors [61—63] for a sequence of irreversible unimolecular reactions which are first-order. We have extended their models by defining each step as a combination of either synthetic or degradative reactions and each step is described by a single rate constant. Also we have allowed for a zero-order synthesis of the first material which can be perturbed, and the use of steady-state approximations. These models and their mathematical solutions are now applicable to the analysis of regulation of enzyme synthesis and degradation. For example, I_1 = mRNA and I_2 = active enzyme as presented by Lee et al. [57].

The integrated solutions as presented in this paper are only functions of the degradative rate constants. These rate constants can be experimentally determined by plotting measured values of ln F_{I_n} versus time. ln F_{I_1} versus time should give a straight line with slope = $-k_1$. ln F_{I_2} versus time should give a curved line with limiting slope = $-k_2$ and intercept of the limiting tangent = $-k_1/k_2 - k_1$, for $k_2 < k_1$.

A limiting slope must be reached or the limiting intercept will have too small a value. The use of labeled compounds facilitates measurements of degradative steps in many systems. Thus, by determining the various rate constants, the behavior of a system over time can be determined explicitly. These models allow description of enzyme levels when the rate of mRNA transcription or the rate of mRNA translation is changed, assuming each is a zero-order process. By adapting these models and their mathematical solutions, it is also possible to describe systems in which other processes are also regulated.

Acknowledgements

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