on day 3, the 16 subjects were rank ordered and divided into two matched groups which would receive the respective extracts. Two hours after the last recipient fish had been tested on day 3, 40 μl of solution was injected intracranially into each of the 16 recipient fish. Eight fish received material from donors trained and then extinguished, and eight fish received material from naive donors. Injections were done according to the method of Agranoff and Klinger (*7*). The 16 fish were injected within approximately 20 minutes.

The 16 recipient animals were given single 20-minute sessions for extinction on days 4, 5, and 6, approximately 19, 43, and 67 hours, respectively, after injection. The testing of the recipient fish was done by an experimenter who was unaware of the group membership of the animals. Two weeks later, the experimental phases from initial testing with light only through final testing of recipients were repeated with 16 additional recipient fish.

The avoidance behavior of the recipient fish was so consistent that matching on the basis of day-3 data also resulted in excellent matching on days 1 and 2 (Fig. 1). A Mann-Whitney U statistic was used to test the differences between the groups on each testing day. The dependent variable analyzed was a difference score, computed for each subject, which consisted of the subject's extinction score (percentage of avoidance) on the day in question minus that subject's acquisition score on day 3. The mean differences were −2.5 and −20.0, respectively, for the control and experimental groups on the first extinction session of the first 16 fish; this was a significant difference ($U = 11$, $n_1 = n_2 = 8$, $P = .014$). For the second 16 fish, the corresponding differences were −8.12 and −26.88 ($U = 11$, $n_1 = n_2 = 8$, $P = .014$). If the two groups were combined, the respective differences would be −5.31 and −23.44, a highly significant difference ($U = 47$, $n_1 = n_2 = 16$, $P < .001$). On days 5 and 6, the difference between groups gradually declined and was no longer significant; this effect is attributable to the extinction of the control group with testing. The performance level of all subjects declined to approximately 35 percent. In this laboratory, performance of fish in various "sensitization" control groups (light only, shock only, unpaired light and shock) is typically around 35 percent after 2 to 13 days under such control conditions. Also, fish given as many as 7 days of extinction training do not extinguish below the 30 to 35 percent level. Thus, 35 percent avoidance responses appears to be a relatively accurate base line, given the particular parameters of this task.

The effect obtained in this study was quantitatively great, statistically significant, and reliable enough to be reproduced in this laboratory. It is not known whether the results are due to an actual transfer of extinction information or to a general facilitation of the extinction process. In any case, one cannot argue that the effect is due to some general activating, exciting, or energizing property of the experimental extract. Of course, one might argue that such an extract exerts a generalized depressing effect on behavior, but then one would have to explain why some extracts activate while others depress.

My results suggest that extinction is a learned reaction to the conditional stimulus which opposes the original reaction conditioned to the conditional stimulus, rather than a simple weakening of the originally acquired reaction. Were the latter the case, one would expect extinction to be characterized by a gradual decrease in the chemical substrate of the conditioned response; it is difficult to conceive of such a lack of substrate from a donor producing a similar lack in a recipient. An interpretation of the extinction process as counterconditioning was also reached recently by Deutsch and Wiener (*8*) in an analysis of extinction through the use of the amnesic properties of physostigmine.

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9. I thank L. Galvan for help in preparing the extracts, and D. Abbott, G. King, R. Mellen, and M. Plummer for assistance during various phases of the experiment.

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Physiological Concentrations of Lactate Dehydrogenases and Substrate Inhibition

Abstract. *Lactate dehydrogenases at physiological concentrations are inhibited by high concentrations of pyruvate when the enzyme and the pyruvate are incubated in the presence of oxidized nicotinamide-adenine dinucleotide before assay. The inhibition is much more pronounced with the H-type than with the M-type lactate dehydrogenase. These results suggest that substrate inhibition may be operative in vivo.*

Substrate inhibition occurs with a number of pyridine nucleotide–linked dehydrogenases. In the case of lactate dehydrogenase (LDH), excess pyruvate significantly inhibits the H-type enzyme under conditions in which the kinetics of the M type are only slightly changed. This difference in substrate inhibition has been suggested as a basis for distinct physiological roles for the two main LDH's (*1*, *2*). The H type may function as a lactate dehydrogenase in heart, whereas the M enzyme may be geared to operate as a pyruvate reductase in voluntary muscle.

The mechanism by which excess pyruvate inhibits LDH may be related to the formation of an abortive ternary complex between pyruvate, nicotinamide-adenine dinucleotide (NAD^+), and the LDH (*2*, *3*). The dissociation constant of this complex is much lower for the H-type enzyme than for the M form. Wuntch *et al.* (*4*, *5*) reported that substrate inhibition may be an artifact of the assay system and hence may not be of any significance in vivo. These conclusions are based on the fact that the H type shows substrate inhibition when the enzyme is diluted, but at high concentrations of enzyme (equivalent to that present in tissues) there is no inhibition. The studies with high concentrations of the LDH were carried out with stopped-flow techniques. Wuntch *et al.* (*4*, *5*) suggest

that possibly a different mechanism is operating when high enzyme concentrations are used.

We report here stopped-flow data indicating that the conclusions reached by Wuntch *et al.* (*4, 5*) are subject to an alternative interpretation and that substrate inhibition is indeed a characteristic of the H catalyst even when large amounts of enzyme are employed.

Our experiments were conducted with an Aminco-Berger stopped-flow apparatus. One syringe contained the reduced coenzyme, and the other syringe contained the enzyme, pyruvate, and oxidized NAD, whenever applicable. The changes in transmission at 340 nm were observed on a Tektronix model 549 storage oscilloscope.

Chicken H_4 and M_4 LDH's were prepared in crystalline form (*6*). The coenzymes were obtained from P-L Laboratories, Inc., and sodium pyruvate was purchased from Sigma. All experiments and incubations were carried out at room temperature.

As pointed out previously, substrate inhibition results with excess pyruvate when some of the NADH is oxidized to NAD+; the resulting NAD+ can then interact with pyruvate and the enzyme to form the abortive ternary complex. There is a time-dependent reaction required for the formation of the complex (*2–4*). With the same amount of NAD+, the time required for formation of the complex will be considerably longer when large concentrations of enzyme are used. With $10^{-6}M$ LDH [the concentration employed by Wuntch *et al.* (*4, 5*)] the complete oxidation of NADH takes place within 0.5 second (Fig. 1). [In the experiments of Wuntch *et al.* (*4, 5*), the same concentrations of NADH were used over the wide range of enzyme concentrations tested.] Hence, the NADH oxidation is complete before any significant amounts of the inhibitory ternary complex can be formed.

There is no difference in rate when $10^{-6}M$ H_4 LDH is employed with either 10^{-2} or $10^{-3}M$ pyruvate (Fig. 1). However, if the enzyme is preincubated with less than $10^{-4}M$ NAD+ and pyruvate, it is readily observed that much more inhibition is associated with the higher ($10^{-2}M$) pyruvate concentration. Inhibition is also observed with $10^{-3}M$ pyruvate when preincubated with the oxidized NAD+.

Table 1 compares the effect of prior incubation with NAD+ on the activities of high concentrations of H_4 and M_4 LDH's. The H type is much more inhibited by the preincubation than is the M-type LDH. These results imply that the difference in substrate inhibition between the two types of LDH also occurs with high concentrations of enzyme as well as with diluted samples of the LDH's.

Table 1. Effect of NAD+ on the activity of high concentrations of chicken LDH. The following concentrations were initially present in the observation chamber: $2.0 \times 10^{-6}M$ enzyme, $7.8 \times 10^{-4}M$ NADH, pyruvate as indicated, $7.8 \times 10^{-5}M$ NAD+, and 0.05*M* tris-(hydroxymethyl) aminomethane-hydrochloride, *p*H 7.5. The NADH was contained in one syringe of the stopped-flow apparatus; all other components were contained in the other syringe.

Additions to LDH	Assay half-time (seconds)	
	H_4 LDH	M_4 LDH
Pyruvate (1 m*M*)	0.12	0.13
Pyruvate (1 m*M*) + NAD+*	0.30	0.17
Pyruvate (10 m*M*)	0.12	0.12
Pyruvate (10 m*M*) + NAD+†	3.00	0.27

* Incubated for 30 minutes before assay. † Incubated for 10 minutes before assay.

In our experiments, the amount of NAD+ in the preincubated mixture was one-tenth of the concentration of NADH used. The rate of formation of the ternary complex is much more rapid when higher concentrations of NAD+ are used.

In all tissues, the concentration of oxidized NAD is higher than that of reduced NAD (*7*). In skeletal muscle, nearly all of the coenzyme is present in the oxidized form. The NAD+ concentration may be close to 1 m*M*. (We used only 0.08 m*M* oxidized NAD.) If skeletal muscle contained the H-type protein, then one might expect a ternary complex to be readily formed with the pyruvate concentrations which are found in muscle. The complex would then act as an inhibitor of pyruvate reduction. It is our thesis that the M type evolved to prevent such a complex from forming in muscle.

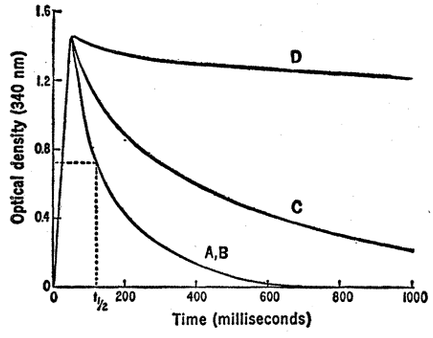

Fig. 1. Typical activity rates of chicken H_4 LDH at various concentrations of pyruvate in the presence and absence of NAD+. The curves represent observed changes in optical density at 340 nm with time. Curve A, $2 \times 10^{-6}M$ enzyme, $7.8 \times 10^{-4}M$ NADH, and $1 \times 10^{-3}M$ pyruvate. Curve B, same amounts of enzyme and NADH and $1 \times 10^{-2}M$ pyruvate. Curve C, same as A, except that the enzyme and pyruvate were preincubated with $7.8 \times 10^{-5}M$ NAD+ for 30 minutes. Curve D, same as B, except that the enzyme and pyruvate were preincubated with $7.8 \times 10^{-5}M$ NAD+ for 10 minutes. All assays were initiated with the addition of NADH.

On the other hand, in aerobic tissues, such as heart, NADH formed from glucose is oxidized by systems other than LDH, and most likely directly or indirectly through mitochondria. To prevent pyruvate from being converted to lactate, the ternary complex of the H-type LDH may have a regulatory role in the metabolism of pyruvate in aerobic tissues. Pyruvate metabolism in heart is normally through oxidation by the mitochondria. However, under anaerobic conditions, in heart, there could be an accumulation of NADH, which could then displace the abortive ternary complex and allow for the reduction of pyruvate to lactate as well as for the oxidation of NADH. We, therefore, suggest that the H-type enzyme has evolved to operate as a regulator of pyruvate metabolism in highly aerobic cells.

It should be pointed out that NADH oxidation in voluntary muscle under anaerobic conditions does not necessarily have to proceed only through LDH. Oxidation could also result from the action of α-glycerolphosphate dehydrogenase. This would lead to the following overall equation:

2 triosephosphate →

pyruvate + α-glycerolphosphate

Hence, pyruvate could accumulate with the NAD still in the oxidized form. Under these conditions, the abortive ternary complex formation would be favored if the H enzyme instead of the M type was present in voluntary muscle.

The degree of ternary complex formation is certainly dependent on the relative ratios of NAD+ and NADH. Manipulation of this ratio, of course, will alter the substrate inhibition, particularly with respect to the H-type LDH. In our initial presentation of our concepts regarding the function of the H and M enzymes, we pointed out the difficulties of translating results in vitro to conditions in vivo. It is our present view, however, that the studies of Wuntch *et al.* (*4, 5*) do not negate our

concepts. We still strongly believe that the H and M types of LDH are geared for functioning in aerobic and anaerobic environments, respectively. To completely prove this point of view, it is important to demonstrate the existence of ternary complexes of the H-type LDH in vivo.

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Production of Urinary Bladder Carcinomas in Mice by Sodium Saccharin

Abstract. *Pellets weighing 20 to 24 milligrams and containing 20 percent sodium saccharin suspended in cholesterol were surgically implanted into the urinary bladder lumens of female Swiss mice (60 to 90 days old) under ether anesthesia. Incidences of mouse bladder carcinomas in animals exposed to these pellets were 47 and 52 percent as compared with incidences of 13 and 12 percent in control mice exposed to pellets of pure cholesterol. The exposure of the mouse bladder to saccharin was very brief, because the time required for 50 percent of the compound to be eluted from the pellets was about 5.5 hours.*

Since the discovery of saccharin (2,3-dihydro-3-oxobenzisosulfonazole) in 1879 (*1*), this chemical has been utilized as a noncaloric sweetening agent in nearly all countries of the world. During the first 6 decades of the 20th century, the use of saccharin in the United States was generally limited to persons with specific medical indications. With the advent of formulations of mixtures of cyclamate and saccharin, increasingly widespread popularity and distribution of a variety of commercial beverages and foods containing these substances occurred (*2*). Thus in 1967, it was estimated (*2*) that nearly 75 percent of the population of the United States consumed some of these nonnutritive sweeteners.

Few studies of the potential carcinogenic activity of saccharin have been conducted in animals (*2*). In one long-term trial of feeding saccharin to rats, it was reported (*3*) in 1951 that "No pathological effect whatever could be attributed to saccharin at levels of 1.0 percent or less. At 5 percent only one effect was noted, in the latter part of the experiment, namely an increased incidence of the ordinarily uncommon condition of abdominal lymphosarcoma. In the 5 percent group, there were seven animals with lymphosarcomas; this number is not out of line with the incidence in comparable groups of rats, but the fact that in four of the seven rats abdominal as well as thoracic lymphosarcomas were present is unusual, since ordinarily the ratio is about 1 to 15-20. Three of these four combinations occurred in animals on experiment one hundred and two or more weeks." No indication that the urinary bladders of these rats were inspected grossly or microscopically was made (*3*). In 1957, Allen *et al.* (*4*), employing the mouse bladder pellet implantation technique in a single experiment involving an evaluation of 13 mice, observed that "Pellets containing saccharin induced a significant incidence of bladder tumours." The mouse bladder carcinogenicity of sodium cyclamate was demonstrated and assayed by the pellet implantation technique (*5*). Oral administration of a mixture of sodium cyclamate and sodium saccharin (10 : 1) at a dose of 2500 mg/day to male and female FDRL Wistar strain rats for a maximum period of 104 weeks was followed by the formation of papillary transitional cell tumors of the urinary bladder in seven males and one female; four to eight of the tumors were diagnosed as carcinomas (*6*). The decisions recently made by public health authorities in several countries of the world, including the United States, Japan, England, Canada, and Sweden, to restrict the consumption of cyclamate, but not restrict the distribution of saccharin, were apparently made (*7*) on the basis of these data (*5, 6*). The observations of Allen *et al.* (*4*) concerning saccharin were apparently not considered because so few animals were evaluated, histopathologic confirmation of diagnoses was absent, and, to our knowledge, this experiment was never repeated. In an attempt to remedy the criticisms of the study of Allen *et al.* (*4*) and to obtain a precise assessment of the mouse bladder carcinogenicity of saccharin, it was retested by the pellet implantation technique.

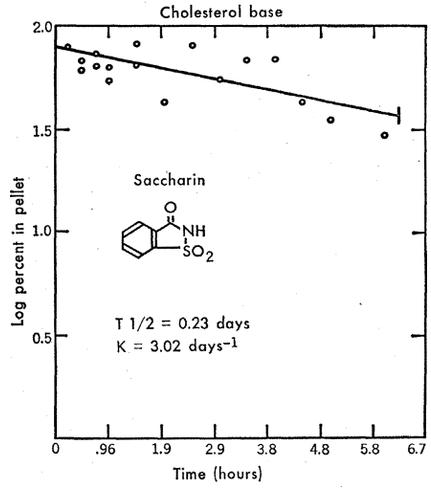

Fig. 1. Elution curve of sodium saccharin from cholesterol.

The experimental details of our use of the above-mentioned technique were reported (*5, 8*). Cholesterol, purified by recrystallization before use, and sodium saccharin (*9*) were ground separately to a fine powder in an agate mortar. The saccharin was mixed carefully with four times its weight of cholesterol before the mixture was compressed into spheroidal pellets, 5/32 inch (0.4 cm) in diameter and 20 to 24 mg in weight, with a standard face die (*5, 8*). The saccharin content of prepared pellets was measured by ultraviolet spectrophotometry (*10*) before use to assure that the quantity of saccharin had not been altered. Prepared pellets were surgically implanted into the urinary bladder lumens of female Swiss mice, 60 to 90 days old (*5, 8*). To estimate the probable exposure of the urinary bladder to saccharin, pellets were removed from some