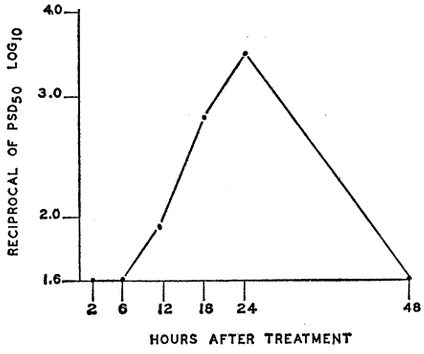

Fig. 1. Appearance of antiviral activity in mouse serum after oral administration of tilorone hydrochloride (250 mg/kg). Activity was measured on mouse L929 cells against vesicular stomatitis virus. First dilution of serum was 1:50.

rum to 0.01 percent ribonuclease for 20 hours at 37°C did not alter its activity. Dialysis of active serum against distilled water at room temperature did not remove the antiviral component. Active mouse serum, diluted in EBME and acidified to *p*H 2.0 with 0.1*N* hydrochloric acid was refrigerated for 24 hours at 4°C. The acid-treated serum, neutralized by dilution in sodium bicarbonate–buffered EBME, retained its activity.

Active mouse serum was compared with serum from untreated mice for inactivation of encephalomyocarditis virus and vesicular stomatitis virus. One to ten dilutions of both serums were prepared in an EBME suspension of each virus. The serum-virus suspensions were incubated for 3 hours at 37°C after which dilutions were made in EBME and applied to monolayers of rabbit kidney cells (RK_{13}). The titer of vesicular stomatitis virus incubated with normal serum was $10^{5.8}$ TCD_{50}/ml and its titer when incubated with active serum was $10^{6.0}$ TCD_{50}/ml. The titer of encephalomyocarditis virus incubated with normal serum was $10^{5.5}$ TCD_{50}/ml and its titer when incubated with active serum was $10^{5.4}$ TCD_{50}/ml. Thus, there was no evidence of virus inactivation by active serum.

If the antiviral component in the serum of mice treated with tilorone is interferon, then serum activity observed in the homologous, mouse L929 cell line should be dependent on the synthesis of new messenger RNA (*2*). A 1:40 dilution of serum with a PSD_{50} of 1:3000, collected from mice treated orally, was incubated for 4 hours at 37°C on confluent monolayers of mouse L929 cells (12 tubes) in the presence of 1.0 μg of actinomycin D per milliliter. After incubation, the cells were washed three times with Hanks balanced salt solution and challenged with 30,000 TCD_{50} of vesicular stomatitis virus. After 1 hour, the cells were washed four times with the Hanks solution to remove extracellular virus; 1.0 ml of EBME was added, and the tubes were incubated again for 20 hours. Groups (12 tubes) of normal serum, normal serum plus actinomycin D, and active serum without actinomycin D were treated similarly. The contents of all tubes within a group were pooled, and virus was titrated by plaque assay. Plaque assay was accomplished by the addition of dilutions (0.2 ml) of virus to confluent monolayers of monkey kidney cells ($LLC\text{-}MK_2$) grown in disposable plastic petri dishes (60 mm by 15 mm; Falcon). After a 2-hour adsorption period at 37°C the cells were washed three times with the Hanks solution to remove extracellular virus. An overlay of a mixture of 0.5 percent methylcellulose and EBME was applied to the monolayers which were incubated at 37°C. Seventy-two hours later, the overlay was removed, the cells were stained with 0.2 percent crystal violet in deionized water, and plaques were counted to determine virus yield. Actinomycin D significantly inhibited the protection normally provided by serum from mice treated orally with tilorone. The virus yield from cells treated with normal mouse serum was $10^{8.1}$ plaque-forming units (PFU) per milliliter, while cells treated with active mouse serum yielded $10^{6.0}$ PFU/ml. The virus yield from cells treated with normal mouse serum in the presence of actinomycin D was $10^{7.6}$ PFU/ml. The virus titer from cells treated with actinomycin D and active serum was $10^{7.5}$ PFU/ml.

We conclude that the antiviral component found in mice treated orally with tilorone is interferon because it (i) is species specific, (ii) has broad antiviral activity, (iii) is inactivated by exposure to a proteolytic enzyme, (iv) is resistant to ribonuclease, (v) is not dialyzable, (vi) is stable to low *p*H, (vii) is not virucidal, and (viii) requires new messenger RNA synthesis for activity as indicated by sensitivity to actinomycin D. Tilorone hydrochloride represents the first recognized synthetic, small molecular weight compound that is an orally active inducer of interferon.

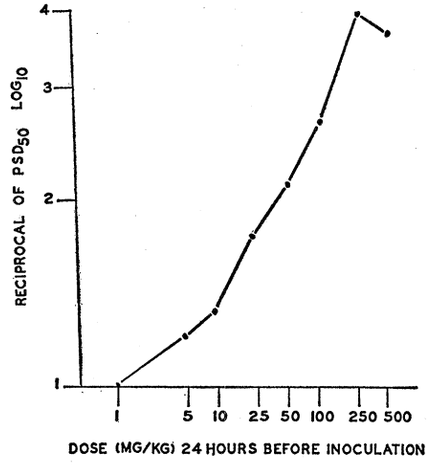

Fig. 2. Quantitative relation of tilorone dose to stimulation of the antiviral serum component in mice. Tilorone was given orally 24 hours before serum collection. Antiviral activity measured against vesicular stomatitis virus (32 TCD_{50}) in mouse L929 cells.

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Enzyme Inactivation with Ultraviolet Laser Energy (2650 Angstroms)

Abstract. *Inactivation of rat heart lactate dehydrogenase was accomplished by irradiation of the enzyme in solution with a frequency quadrupled neodymium glass laser.*

Laser energy has shown biological and biochemical activity in the visible region of the spectrum. Due to the fact that only a few natural chromophores are available for absorption of the visible wavelengths, the biological effectiveness of lasers has been limited to studies that made use of these

chromophores or utilized vital dyes (*1, 2*). While ruby laser energy at 6943 Å has been reported to affect peroxidase activity (*3*) few other enzymes have responded. Even with the doubled ruby laser frequency at 3471.5 Å, enzymatic activity was affected only after an appropriate chromophore absorbed the laser energy and transferred it to the enzyme molecule (*4*). The absorption characteristics of nucleic acids and proteins suggest that the fourth harmonic of the neodymium laser (*5*) would be absorbed sufficiently well to affect the biochemical activity of these molecules. A preliminary survey was made with such a device in order to determine whether this hypothesis could be verified.

The laser system consisted of a neodymium doped, Q-switched laser (Korad, model KIQP) with a primary output of 125 Mw in the infrared region of the spectrum (10,600 Å), and half pulse width of 15 nsec. A potassium dihydrogen phosphate crystal was inserted in the cavity to act as a frequency doubler to generate the second harmonic at a wavelength of 5300 Å with an efficiency of 8 to 10 percent. This green light was transmitted through a mirror which was 100 percent reflective to the infrared and was passed through a second doubling crystal with a conversion efficiency of 1 to 2 percent. The converted ultraviolet wavelength at 2650 Å was transmitted through a quartz filter, which was 100 percent absorbant for the 5300 Å. Utilizing the malachite green leucocyanide actinometric technique, described by Fisher *et al.* (*6*), we found that a power density of 150 to 180 kw of the far ultraviolet wavelength was emitted in a 15-nsec pulse width. A helium-neon alignment laser was utilized to indicate the position of the ultraviolet beam in order to properly orient the target.

The enzyme system selected for this preliminary study was lactate dehydrogenase (LDH). The oxidation of reduced nicotinamide adenine dinucleotide (NADH), in the presence of pyruvate and LDH, was used as an assay system. The stock substrate solution contained 0.4 mg of pyruvate and 0.4 mg of NADH per milliliter of balanced salt solution. The stock enzyme solution contained 9.2 international units (I.U.) of lactate dehydrogenase per milliliter of balanced salt solution. To run a reaction, 0.05 ml of the LDH stock (0.46 I.U.) was placed in a quartz spectrophotometer cuvette, and 2 ml of the stock substrate solution was added. The cuvette was then vigorously agitated for 1 to 2 seconds, placed in a recording spectrophotometer, and the change in absorbance at 3400 Å was recorded over a period of several minutes. The change reflected the conversion of the NADH (with an absorption maximum at 3400 Å) to the oxidized form. The rate at which the reaction occurred reflected the concentration of the enzyme.

Table 1. Time for reaction (conversion of NADH to NAD) to reach completion.

Before laser controls (sec)	Laser-irradiated samples		After laser controls (sec)
	Duration (sec)	Pulses (No.)	
	Experiment 1		
150	255	30	165
155			150
150	585	60	
	Experiment 2		
150	255	30	150
155	270	30	150
135	540	60	155
	Experiment 3		
165	255	30	150
135	270	30	

After establishing a control enzymatic reaction rate at room temperature, a portion of the enzyme (0.05 ml) was placed in a quartz spectrophotometer cuvette and positioned in the path of the ultraviolet laser beam. The laser beam was focused with a quartz lens through the front surface of the cuvette into the LDH solution. The enzyme samples received from 30 to 60 laser pulses with a repetition rate of 45 seconds between pulses. Immediately after the last exposure of the enzyme to the laser power, 2 ml of the substrate solution was added to the cuvette, and the rate of NADH oxidation was monitored spectrophotometrically. An additional unirradiated sample of LDH that was maintained at the same temperature as the irradiated sample was evaluated for catalytic activity after the enzyme samples were laser irradiated.

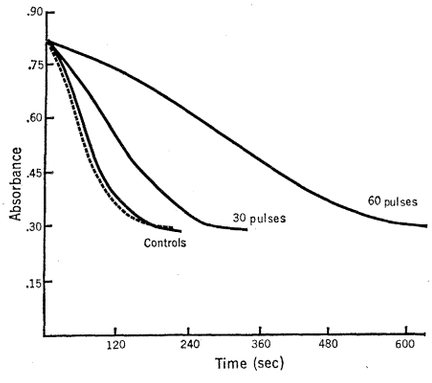

Fig. 1. Rate reaction curve of the conversion of NADH to NAD in the presence of pyruvate and lactate dehydrogenase. The absorbance readings were taken at 3400 Å. Lactate dehydrogenase samples received 30 to 60 pulses from the quadrupled neodymium laser. The control samples of LDH did not receive laser irradiation; the dashed curve was a control sample run prior to the laser irradiation, and the solid curve was a control run after the laser irradiation.

The results are illustrated in Fig. 1 and Table 1. The figure illustrates typical rate reactions for "before" and "after" control samples as well as for samples that were irradiated with 30 to 60 pulses. Table 1 presents data from several replicate experiments.

It was apparent that the ultraviolet laser power affected the enzymatic activity of LDH. Furthermore, it appeared that the degree of inactivation was proportional to the amount of ultraviolet radiation power imposed, since the portion that received 60 pulses took approximately twice as long to run to completion as the portion that received 30 pulses.

An effort was made to determine whether the laser irradiation altered the mass or the electric charge of the enzyme molecule. Two irradiated (60 pulses) and two control samples were subjected to gel electrophoresis and nitrotetrazolium blue–reaction staining for the LDH isozymes. The five isozyme bands migrated to the same distance in both control and irradiated preparations, indicating a lack of physical change to these enzyme molecules. The irradiated samples showed only a weak staining reaction, which suggests some structural change of the molecule; however, the data were insufficient to develop an interpretation of this effect. In earlier studies it was reported that the activity of LDH was not affected by radiation (ultraviolet and x-ray) but that the electrophoretic mobility was affected (*7*). Our results are to the contrary. The nature of the difference between the ultraviolet laser effects and the effects obtained with other radiation sources remains to be determined.

While the mechanism of action of the far ultraviolet laser on the enzyme molecule has not yet been determined, it is apparent that this laser power can affect enzymatic activity. Biological studies on tissue culture cells are being performed with this laser system to consider the in vivo influence of the

2650-Å wavelength on enzymes, as well as the theoretical effect on nucleic acids. In addition, the short pulse duration and high energy densities of this and more advanced laser systems are opening up several areas to the photochemist: studies on transient changes, biphotonic processes, intersystem crossing to the triplet state, and possibly even studies on the lifetimes of excited states of proteins and nucleic acids.

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Polypeptide with Broad Biological Activity: Isolation from Small Intestine

Abstract. *A polypeptide, which has potent and diverse biological action—including systemic vasodilation, hypotension, increased cardiac output, respiratory stimulation, and hyperglycemia—was isolated from the small intestine of the hog. The peptide has 28 amino acid residues and is chemically distinct from the kinins, "substance P," glucagon, and secretin.*

Polypeptide hormones regulate many physiologic functions and mediate certain pathologic responses. We report here the isolation of a new polypeptide possessing an unusually wide range of biological activity affecting cardiovascular, respiratory, and metabolic functions. The peptide, extractable from small intestine, causes systemic vasodilation, hypotension, increased cardiac output, stimulation of respiratory chemoreceptors, and hyperglycemia. Chemically, it has an amino acid composition that distinguishes it from other naturally occurring peptides with similar actions, such as the kinins, "substance P," and glucagon.

The peptide was prepared from the starting material (methanol extract of hog small intestine) used by Jorpes and Mutt for the preparation of secretin (*1*). The purification procedures consisted of two steps of ion-exchange chromatography, counter-current distribution, and gel chromatography. On chromatography on carboxymethyl cellulose in 0.0125*M* phosphate buffer, the active fraction was retained on the column but eluted with 0.2*M* HCl. It was chromatographed again on carboxymethyl cellulose in 0.1*M* ammonium bicarbonate. The active material emerged from the column late but with the same buffer. It was then subjected to counter-current distribution in a system of 1-butanol and 0.1*M* NH_4HCO_3. After a 200-tube transfer, the material in tubes 60 to 85 was recovered and a portion of it was hydrolyzed and analyzed for amino acids by two-dimensional paper chromatography (*2*). Two amino acids, glycine and proline, were present in much smaller amounts than any of the other amino acids found. The bulk of the material was then chromatographed in 0.2*M* acetic acid on Sephadex G-25 (Pharmacia). On hydrolysis and quantitative amino acid analysis (*3*), the active fraction was found to contain no proline and only a trace of glycine (less than one-tenth, on a molar basis, that of any other amino acid present). Tryptophan and cysteine/cystine were absent, as determined by the Voisnet-Rhode dimethylaminobenzaldehyde reaction (*4*) and by quantitative analysis of material that had been oxidized with performic acid before acid hydrolysis (*5*), respectively. The quantitative amino acid analysis suggested a polypeptide with 28 residues, having the following composition: 2 alanine, 5 aspartate/asparagine, 2 arginine, 1 glutamate/glutamine, 1 histidine, 1 isoleucine, 3 leucine, 3 lysine, 1 methionine, 1 phenylalanine, 2 serine, 2 threonine, 2 tyrosine, and 2 valine.

All ratios for the molar quantities of the amino acids were reasonably near whole numbers, indicating that the polypeptide was in an essentially pure form. This was supported by the appearance of one band on electrophoresis in polyacrylamide gel (*6*) and the finding of only one *N*-terminal amino acid, histidine, by the Edman method (*7*).

The absence of glycine and proline residues distinguishes this newly isolated peptide from other vasoactive peptides, the kinins (*8*) and "substance P" (*9*). Like secretin (*10*) and glucagon (*11*), the new peptide has an *N*-terminal histidine residue, but the lack of glycine and the presence of isoleucine clearly set it apart from these two hormones.

Investigation of the biological effects of the peptide was carried out in dogs anesthetized with pentobarbital or with chloralose and urethane. Blood flow in ascending aorta (cardiac output) and other systemic arteries was measured by noncannulating electromagnetic flow probes and flowmeter (Carolina Medical Electronics). Arterial blood pressure was recorded by Sanborn 267B transducers connected to an intra-aortic catheter, and respiratory minute volume was monitored by electronic integration of the signal from a pneumotachygraph attached to the airway. The concentration of glucose in the blood was estimated by a hexokinase-coupled enzymatic method.

Bioassay was based on the systemic vasodilator action reported for partially purified fractions (*12*). Assays were repeated in the same animal and in numerous animals. Intra-arterial infusion of the pure peptide at doses of 40 ng/kg increased femoral arterial flow by 50 percent. Larger doses (400 ng/kg) tripled blood flow and kept it above normal for 27 minutes. Local injection also increased superior mesenteric arterial flow (34 percent), but renal arterial flow did not change. On intravenous infusion of the latter dose, mean systemic arterial blood pressure fell by 15 mm-Hg, and total cardiac output increased by 43 percent ($P < .001$). The increase in cardiac output was due more to a greater (29 percent) stroke volume than to a faster (8 percent) heart rate. A possible direct myocardial action has not been established.

Respiratory minute volume was augmented by 30 percent, the hyperpnea resulting both from an increased frequency and a larger tidal volume. To determine the mechanism of this hyperventilation, we infused the peptide into one common carotid artery before and