pathway of catabolism (*12*). However, the precise molecular role of 24,25-$(OH)_2D_3$ remains to be elucidated.

These results are consistent with several inferential reports of positive biological contributions of 24,25$(OH)_2D_3$. Miravet *et al.* (*13*) found that 24,25-$(OH)_2D_3$ administration to phosphate-depleted, vitamin D–deficient rats resulted in a decrease in serum calcium levels. Also, Bordier *et al.* (*14*) reported that simultaneous oral administration of 1,25-$(OH)_2D_3$ and 24,25$(OH)_2D_3$ to human sugjects with vitamin D–deficient osteomalacia was more effective than 25-$(OH)_2D_3$ or 1,25$(OH)_2D_3$ alone in stimulating the rate of bone mineralization. Similarly Kanis *et al.* (*15*) reported preliminary evidence in a limited number of normal subjects and patients with chronic renal failure that short-term oral administration of 24,25$(OH)_2D_3$ stimulated intestinal calcium absorption and reduced serum alkaline phosphate levels; this would be consistent with an effect of 24,25$(OH)_2D_3$ on bone mineralization. When these reports (*13–15*) are taken in concert with our previous report concerning the effectiveness of simultaneous dosing of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ on parathyroid gland reduction (*7*) and our present data, it seems clear that 24,25$(OH)_2D_3$ is an integral component, along with 1,25$(OH)_2D_3$, of the vitamin D endocrine system.

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9. After the age of 22 weeks the hens were housed in individual cages designed for egg collection. Dietary calcium was increased to 3.0 percent and the lighting cycle was changed to 12 hours of light and 12 hours of darkness (from 9 and 15 hours, respectively).
10. Hatching of viable chicks normally occurs after 21 to 22 days of incubation. The chicks in this experiment hatched between 21 and 23 days. After 23 days, chicks in eggs that had not hatched were assumed to have died from lack of air and the eggs were frozen for later analysis. These chicks showed no gross structural abnormalities that could explain their failure to hatch. When candled at 20 days of incubation these chicks all appeared to be normal and alive and could not be distinguished from those chicks which subsequently hatched successfully. It is interesting to note that failure to hatch was absolute in that no pipping (initial shell breakage) occurred. Whether these chicks died prior to pipping or were unable to pip is not clear.
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Phenylalanine Depletion for the Management of Phenylketonuria: Use of Enzyme Reactors with Immobilized Enzymes

Abstract. *Multitubular enzyme reactors with immobilized phenylalanine ammonia lyase were tested in vitro and in vivo for depletion of phenylalanine in circulating blood. Sustained reduction of phenylalanine was achieved in less than 30 minutes. A 50 percent decrease of phenylalanine was obtained with a 2-hour application of enzyme reactors and was maintained for more than 2 days. Similar enzyme reactors have therapeutic potential for temporary management of phenylketonuric patients when their circulating phenylalanine becomes exceedingly high because of infection, fever, or pregnancy.*

A genetic deficiency of phenylalanine hydroxylase, an enzyme that degrades phenylalanine, is responsible for the clinical entity of phenylketonuria (PKU), a syndrome characterized by high levels of circulating phenylalanine. The degree of neuropsychiatric disorders, such as mental retardation, confusion, and irritability, correlate with the amount of phenylalanine in the blood (*1*). In addition, injection of phenylalanine into normal volunteers decreases their mental acuity and coordination (*2*). Even when symptoms in PKU patients are controlled by a phenylalanine-poor diet, phenylalanine in their blood rises during episodes of fever and infection (*3*). Increase in circulating phenylalanine has also been reported during pregnancy, resulting in mental retardation in genetically normal children because of their exposure in utero to the high concentrations of phenylalanine (*4*).

Treatment of PKU patients with replacement of phenylalanine hydroxylase does not seem practical because of the

Table 1. Metabolism of L-phenylalanine by phenylalanine ammonia lyase reactors, measured in 120 ml of blood circulating in vitro at a flow rate of 100 ml per minute at 37°C. Blood phenylalanine was assayed enzymatically (*8*) by measuring the rate of degradation by a standard amount of lyase reactor. Results are expressed as the percent reduction of phenylalanine in times given. Base line determinations were made before the addition of 30 mg phenylalanine per 100 ml of blood. The unit of activity of the lyase reactors is the number of micromoles of phenylalanine metabolized per minute when a 1 m*M* phenylalanine solution in 50 m*M* borate buffer, *p*H 8.5, is circulated through the reactor at a flow rate of 50 ml/min at 25°C.

Reactor		Percentage of circulating phenylalanine metabolized			
Series	Units of activity	Base line at 10 minutes	After phenylalanine added		
			5 minutes	10 minutes	2 hours
HP-303	100	22	78	88	91
HP-304	10	33	83	84	90
HP-311	90	25	67	84	86
HP-312	180	19	83	83	86

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Table 2. Relation between flow rate and volume of in vitro circulating blood to depletion of its phenylalanine content by phenylalanine ammonia-lyase reactor HP-304.

Flow rate (ml/min)	Circulating volume (ml)	Time for one circulation (minutes)	Percentage of phenylalanine metabolized in	
			5 minutes	Ten circulations
100	120	1.2	84	84 (12 minutes)
100	240	2.4	63	84 (24 minutes)
240	200	0.8	84	84 (8.3 minutes)

difficulties in isolation and purification of the enzyme in large quantities from mammalian tissues, and because of its short half-life in the circulation. Another phenylalanine-metabolizing enzyme, phenylalanine ammonia-lyase, is readily available from microbial sources; however, it is highly antigenic and is rapidly cleared from the circulation, particularly after repeated administration (*5*). In theory, immobilized enzymes are not metabolized to a significant degree and thus small quantities can be used repeatedly. Immunological reactions against immobilized antigens are also expected to be minimal. We have previously explored the ability of other immobilized enzymes to activate circulating substrates (*6*). We also reported on the immobilization of phenylalanine ammonia-lyase on the inner wall of multitubular enzyme reactors to be placed in an extracorporeal shunt for the breakdown of phenylalanine in the blood (*7*). We now report some in vitro and in vivo experiments with phenylalanine ammonia-lyase reactors in which we explored their potential for temporary reduction of phenylalanine in PKU patients.

Reactors were assigned arbitrary units of activity (Table 1) (*7*), and those with an activity of ten or greater were tested by continuously recirculating dog blood with a peristaltic pump. The blood reservoir and reactor were maintained at 37°C. Blood phenylalanine was measured (*8*) during 10 minutes of circulation through the reactor; at that time 30 mg of phenylalanine was added for every 100 ml of blood, and circulation continued for two additional hours.

Various types of reactors prepared with nylon tubes were compared (Table 1). Initial levels of phenylalanine varied between 2.3 and 4.8 mg per 100 ml of blood. Only 19 to 33 percent of endogenous phenylalanine was degraded in 10 minutes of exposure to immobilized phenylalanine ammonia-lyase. However, 67 to 83 percent of the added phenylalanine was removed in 5 minutes, and base line levels were achieved after 10 minutes. Continued circulation through the reactors for up to 2 hours caused no further decrease in the phenylalanine concentration. No significant difference was observed between the depleting capacity of the reactors, despite significant variation in their activity. Even the least active reactor (HP-304) was able to degrade 83 to 88 percent of the added phenylalanine (19 to 25 mg) in 5 minutes.

In other experiments (Table 2), we varied the flow rate and volume of circulating blood passing through reactor HP-304 to assess its performance for in vivo application. Under these conditions, flow rate and volume seemed to have little effect on the phenylalanine-removal capacity of the reactors. Such results would be expected when the rate of the reaction is kinetically controlled (*6*). A slow rate of flow through the reactors did not affect the activity of the immobilized phenylalanine ammonia-lyase. No loss of activity was observed due to deposition of proteins on the inner surface of the reactor or to circulating inhibitors.

The phenylalanine ammonia-lyase reactors were tested in vivo by incorporating them into a shunt between the femoral artery and vein of the heparinized dogs. Flow through the reactor was maintained with a peristaltic pump at 100 ml/min, with occasional adjustments to keep the blood pressure constant. Blood pressure and other vital signs were monitored throughout the experiment.

Several methods were used to produce sustained, high circulating phenylalanine in the dogs. When phenylalanine was injected into normal animals (Fig. 1), no significant difference was observed in the initial decrease and the sustained phenylalanine level achieved between animals injected intravenously with 21 mg of phenylalanine per kilogram body weight and the animals which, in addition, were infused with 21 mg of phenylalanine per kilogram of body weight per hour. Since

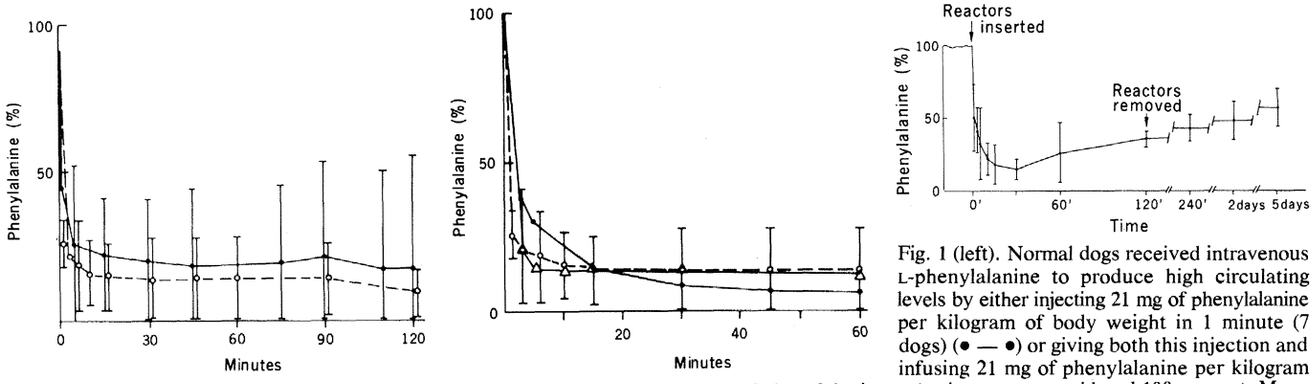

Fig. 1 (left). Normal dogs received intravenous L-phenylalanine to produce high circulating levels by either injecting 21 mg of phenylalanine per kilogram of body weight in 1 minute (7 dogs) (• — •) or giving both this injection and infusing 21 mg of phenylalanine per kilogram of body weight per hour, starting at the same time (4 dogs) (○ --- ○). The amount of phenylalanine at 1 minute was considered 100 percent. Mean ± 95 percent confidence limits are expressed. Fig. 2 (middle). Normal dogs received 21 mg of phenylalanine per kilogram of body weight, intravenously, in 1 minute, plus an infusion of 21 mg of phenylalanine per kilogram of body weight per hour. Shunting of circulation through the reactor occurred after the injection (0 time), and phenylalanine at this time was considered 100 percent. Mean phenylalanine levels are plotted against time, and 95 percent confidence limits are indicated. Control group, ○ --- ○; HP-315 reactor (18 activity units), • — •; HP-312 reactor (180 activity units), △ — △. Fig. 3 (right). Change in blood L-phenylalanine (mean ± 95 percent confidence limit) with phenylalanine ammonia-lyase reactors in dogs having experimental PKU. The percent of initial phenylalanine varied among the dogs from 11 to 29 mg per 100 mg of blood. Reactors were inserted into an extracorporeal shunt established between the femoral vein and femoral artery. Experimental PKU was produced by feeding the dogs 200 mg of phenylalanine per kilogram of body weight daily and 100 mg *p*-chlorophenylalanine per kilogram of body weight every 3 days for 4 weeks.

the 95 percent confidence limits were narrower for the second group, this dose schedule was employed for testing the lyase reactors.

The change in circulating phenylalanine levels produced by reactors with low (HP-315) and high (HP-312) activity were compared (Fig. 2). Because of the sudden initial decrease of phenylalanine and the large variation among controls, it was difficult to evaluate further changes produced by the reactors. Under the conditions of these experiments, the phenylalanine levels obtained with the reactors overlapped those of the control group.

We therefore employed a different method to produce sustained hyperphenylalaninemia in dogs. We adapted to dogs and monkeys a method previously used in rats (*9*) to induce experimental PKU. We administered orally 200 mg of phenylalanine per kilogram of body weight daily, and 100 mg of *p*-chlorophenylalanine (a phenylalanine hydroxylase inhibitor) per kilogram of body weight every third day, resulting in hyperphenylalaninemia within 3 to 4 weeks. With this method it was possible to raise circulating phenylalanine to 11 to 29 mg per 100 ml of blood. The variation among animals was of no major concern, since, in the assay employed, each animal served as its own control.

Multitubular lyase reactors were inserted into arteriovenous shunts placed in heparinized dogs with experimental PKU (Fig. 3). Temporary shunts were prepared on both sides of four animals. Because of the variation among animals, phenylalanine is expressed as the percentage of initial values obtained before application of the reactor. After 15 minutes of exposure to the reactor, circulating phenylalanine decreased to 18 percent of the initial concentration. After the animals were treated for 30 minutes, phenylalanine started slowly to rise, but stayed below 40 percent of the initial value. After the reactor was removed, the rise continued, but was still approximately 50 percent 5 days after reactor application.

The rise within hours after sudden depletion is probably due to the release of phenylalanine from erythrocytes and tissues, in order to reestablish the equilibrium between circulating and stored phenylalanine. The later, slow rise of phenylalanine is the result of continuous dietary administration of phenylalanine and *p*-chlorophenylalanine.

On the basis of these experiments, it seems feasible to remove blood phenylalanine by multitubular enzyme reactors. Suitable enzyme reactors could become clinical tools for the management of PKU patients, particularly at times of infection and pregnancy-induced exacerbation.

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Tail Pinch Versus Brain Stimulation: Problems of Comparison

Koob *et al.* (*1*) claim that, since tail pinch and brain stimulation produce behaviors with common properties, "both manipulations may act through the same mechanism." This conclusion is not warranted by the authors' experiments. The results were interpreted in terms of qualitative similarities between the behavioral effects of electrical stimulation of the hypothalamus and tail pinching with a paper clip. However, the fact that the two types of stimulation can induce behaviors with common properties in no way constitutes evidence that they exert their behavioral effects through a common mechanism. An additional difficulty is that it is impossible to determine the magnitude of the effect of tail pinch, since unpinched control animals were not included. Certainly, wood-gnawing and eating have measurable latencies and durations in unpinched rats, and such measurements should have been reported.

Nevertheless, Koob *et al.* demonstrated that while the tail of a rat is being pinched, behaviors with the following properties occur. (i) The nature of the behavioral response is somewhat arbitrarily determined by goal objects. (ii) The response changes gradually with latencies decreasing and durations increasing over time. (iii) New habits will be learned in order to execute responses during tail pinching. The authors argue that these characteristics apply to the behavioral effects of both tail pinch and electrical stimulation of the brain. We agree that these characteristics may apply to behaviors produced by generalized behavioral activation. We are equally certain that arousal or generalized activation can be produced by electrical stimulation of certain brain regions. However, we are complelled to point out that electrically elicited behaviors cannot be universally accounted for in terms of arousal-related processes.

With regard to the first two points, critical distinctions between the behavioral effects of brain stimulation and responses to generalized arousal are evident from research over the past several years in which we have electrically stimulated the hypothalamus in rhesus monkeys free to move and interact socially during stimulation.

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