Relevance of Free Tryptophan in Serum to Tissue Tryptophan Concentrations

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The consumption of a carbohydrate diet by fasted rats is followed by major decreases in serum nonesterified fatty acids (NEFA) and nonalbumin-bound tryptophan (unbound tryptophan), but by increases in serum total tryptophan and brain tryptophan; the tryptophan concentrations of liver and small intestine are unchanged, while that of skeletal muscle falls slightly. The addition of 15% or 30% fat to a protein–carbohydrate diet results in dose-related increases in serum NEFA and serum unbound tryptophan, but no significant changes in serum total tryptophan or brain tryptophan. The observation that diet-induced changes in serum unbound tryptophan does not correlate with brain tryptophan concentrations is independent of the method used to separate free from albumin-bound serum tryptophan. These studies confirm that, in the rat, a major physiologic regulator of the extent to which serum tryptophan binds to albumin is the concentration of NEFA in serum. These studies also provide additional evidence that the concentration of tryptophan in the brain is not necessarily determined by the size of the unbound pool of tryptophan in blood as measured in serum.

TRYPTOPHAN CIRCULATES in blood in two forms, i.e., bound to albumin and unbound.[1] The proportion of tryptophan that is bound is largely controlled by plasma nonesterified fatty acid (NEFA) concentrations. NEFA interfere with the binding of tryptophan to albumin; hence increases in NEFA cause bound tryptophan to be released into the unbound or "free" pool.[2-4] The rates at which certain biologically active compounds pass from media into tissues are retarded when these compounds are bound to plasma proteins.[5] On the basis of such effects and of parallel changes that occurred in brain tryptophan and plasma free tryptophan after various treatments, it was suggested that albumin binding similarly retards the entry of tryptophan into the brain, and that plasma free tryptophan (and not total tryptophan) is the best predictor of changes in brain tryptophan.[6-8] In contrast, we have suggested that brain tryptophan varies directly with the total plasma tryptophan concentration and inversely with the plasma concentration of other neutral amino acids (e.g., leucine, tyrosine) that compete with tryptophan for entry into the brain.[9,10]

In a preliminary study in which rats were given access to a carbohydrate diet that decreased the concentration of unbound tryptophan, it was found that brain tryptophan levels *increased*.[2] Thus, it appeared that, following physiologic

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treatments such as food consumption, brain tryptophan and plasma free tryptophan did not necessarily change in parallel. In the present study serum unbound tryptophan has been varied over a wide concentration range by allowing rats to consume diets of differing composition, and the relationships between the concentrations of unbound tryptophan in the blood and tryptophan in the brain have been explored. Our observations indicate that the changes in serum unbound tryptophan occurring acutely in response to the consumption of diets of defined composition are, in general, either opposite in direction to or unaccompanied by changes in tissue tryptophan levels.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) weighing 150–200 g were maintained at 20–22° C in animal quarters illuminated from 9 a.m. to 9 p.m. by "Vita-Lite" (50–75-ft candles; Duro-Test Corporation, North Bergen, N.J.). The animals were deprived of food but not water at 5 p.m. in the evening before an experiment. Starting at 10 a.m. the following morning they were given access to food and water for the duration of the experiment (1–4 hr); the subsequent food consumption was measured. Control animals continued to receive water only. Control and fed animals were decapitated alternately, in order to minimize effects due to time lapses involved in killing large batches of animals. Blood from the cervical wound was collected in tubes maintained at 0°C, and the serum was harvested by centrifuging at 800 *g* for 30 min. The tubes were stored at −20°C in an atmosphere of 95% N_2–5% CO_2 to ensure that the pH of their contents would be maintained at near physiologic levels. The concentrations of serum unbound tryptophan were determined by equilibrium dialysis at 37°C for 3.5 hr at pH 7.5.[1,3] In one study, serum free tryptophan was also measured after equilibrium dialysis at 0°C for 24 hr (at which time equilibrium is achieved) and after ultrafiltration using Centriflo CF-50A cones (Amicon Corp., Lexington, Mass.). For ultrafiltration, 1 ml of serum was pipetted into the cones and centrifuged for 30 min at 800 *g* in an International Centrifuge at room temperature. Free tryptophan in the ultrafiltrate (0.100 ml) and total tryptophan (0.025 ml) of untreated sera samples were assayed fluorimetrically.[11] The cones were prewashed with water, and the washings were tested for substances that might fluoresce at the same wavelength as the norharman product of the tryptophan assay; no impurity was detectable.

Further studies on the ultrafiltration technique indicated that the concentrations of serum free tryptophan obtained thereby varied with the volume of serum used, and to a lesser extent with the *g* force and the time of ultrafiltration. Recentrifugation of serum samples greatly increased their apparent free tryptophan content. Additionally, both temperature and pH, both important factors in binding, are difficult to control. Therefore, this method was not routinely used.

Brains were removed immediately after decapitation, transected midsagitally, and frozen at −70°C. Tryptophan was assayed using one-half brain by the following modification of the method of Denckla and Dewey:[11] After homogenization for 10 sec in 5 volumes of HCl (10^{-4} *M*) with a Polytron homogenizer (Brinkman Corp., Westbury, N.Y.), an aliquot (0.05 ml) was added to 1.8 ml HCl (10^{-4} *M*) and protein was precipitated with 75% trichloracetic acid, 0.2 ml. After centrifugation ferric chloride · $6H_2O$, 0.2 ml (0.073 g in 5 ml formaldehyde + 95 ml H_2O) was introduced to the reaction vessel in a darkened room, which was maintained darkened for the rest of the procedure.[12] The recovery of added tryptophan was greater than 95%. Brain serotonin and 5-hydroxyindoleacetic acid were measured fluorimetrically in the other half-brain.[13]

Muscle (quadriceps), liver, and small intestine (the terminal 30 cm of ileum, well washed with .9% saline) were also stored at −70°C and assayed for tryptophan as described above. All tissues were assayed together within 2 days after the experiment.

Serum NEFA were assayed titrimetrically.[14] Liver tryptophan pyrrolase (L-tryptophan: oxygen, oxidoreductase, EC 1.13.1.12) was assayed with and without added methemoglobin and ascorbic acid.[15,16]

RESULTS

Effect of a Carbohydrate Meal on Serum Unbound Tryptophan

Serum NEFA were significantly lower among animals killed 1 or 2 hr after the onset of carbohydrate consumption than among fasted control rats (Table 1). During this interval, the concentration of unbound tryptophan in the serum declined in parallel with that of NEFA; the percentage of total tryptophan represented by the unbound moiety fell from 28%–30% to 17% at 1 hr, and then to 13% at 2 hr (Table 1). Although the concentration of serum unbound tryptophan declined to half the fasting value, serum total tryptophan rose, indicating that the reduction in unbound tryptophan was probably a consequence of enhanced association of the amino acid with albumin. The concentration of tryptophan in skeletal musle fells by 13% after 2 hr ($p < 0.05$), whereas liver and smooth-muscle tryptophan concentrations remain unchanged, and brain tryptophan levels rose by 24% ($p < 0.05$). Subsequent experiments confirmed the existence of a small decline in muscle tryptophan concentration at various times up to 4 hr after initiation of carbohydrate consumption; however, only at 2 hr was this reduction statistically significant. Tryptophan pyrrolase activities in livers (assayed with and without methemoglobin and ascorbic acid) taken from fasted animals did not differ significantly from activities in livers of animals given access to carbohydrate for 2 or 4 hr. Hence, major changes in

Table 1. Effect of Carbohydrate Consumption on Serum Free Tryptophan and Tissue Tryptophan Concentrations

	One Hour		Two Hours	
	Fasted	Fed	Fasted	Fed
Serum NEFA	0.945 ± 0.073	0.404 ± 0.033*	0.879 ± 0.080	0.337 ± 0.041*
Serum total tryptophan μg/ml	14.6 ± 0.7	16.6 ± 0.5†	15.8 ± 0.6	17.5 ± 0.5‡
Serum unbound tryptophan μg/ml	4.1 ± 0.2	2.8 ± 0.2*	4.8 ± 0.2	2.3 ± 0.2*
(% of total)	28%	17%	30%	14%
Brain tryptophan μg/g	3.4 ± 0.3	3.7 ± 0.2	3.7 ± 0.2	4.6 ± 0.3†
Muscle tryptophan μg/g	6.0 ± 0.2	5.4 ± 0.4	7.0 ± 0.3	6.1 ± 0.2
Liver tryptophan μg/g	22.5 ± 1.8	24.6 ± 1.0	27.8 ± 2.0	24.0 ± 1.8
Small intestine tryptophan μg/g	—	—	16.0 ± 0.6	16.3 ± 1.1

Four groups of animals (12 rats per group) were fasted overnight. At 9:30 a.m. to 11:30 a.m. two groups were given access to a carbohydrate diet[2] containing no fat. Controls had free access to water but not food. Animals were killed alternately at 1 and 2 hr following food consumption. Results in this and Tables 2–4 are expressed as mean ±SEM. Each rat ate an average of 6 g in the first hour and 2 g in the second hour.

*$p < 0.001$, differs from corresponding fasted controls.

†$p < 0.05$, differs from corresponding fasted controls.

‡$p < 0.02$, differs from corresponding fasted controls.

serum unbound tryptophan (Table 1) were not associated with alterations in this substrate-inducible[17] enzyme.

Effect of Dietary Fat on Serum Unbound Tryptophan and Tissue Tryptophan

To determine whether dietary fat modifies the binding of circulating tryptophan to rat albumin in vivo, groups of fasted rats were allowed to consume complete diets containing casein (20%) and various amounts of fat (0%, 15%, 30%, or 45%) and carbohydrates; the animals were killed 2 hr after food was presented. The postprandial NEFA concentration reflected the proportion of fat in the diet, i.e., it varied from 0.260 meq/liter in animals consuming the nonfat meal, to 1.8 meq/liter in those consuming the 45% fat meal (Fig. 1). This latter concentration is considerably higher than that normally found in fasting animals (Table 1).

Total serum tryptophan levels did not differ in animals eating diets containing 0%, 15%, or 30% fat, but were depressed by about 15% in animals consum-

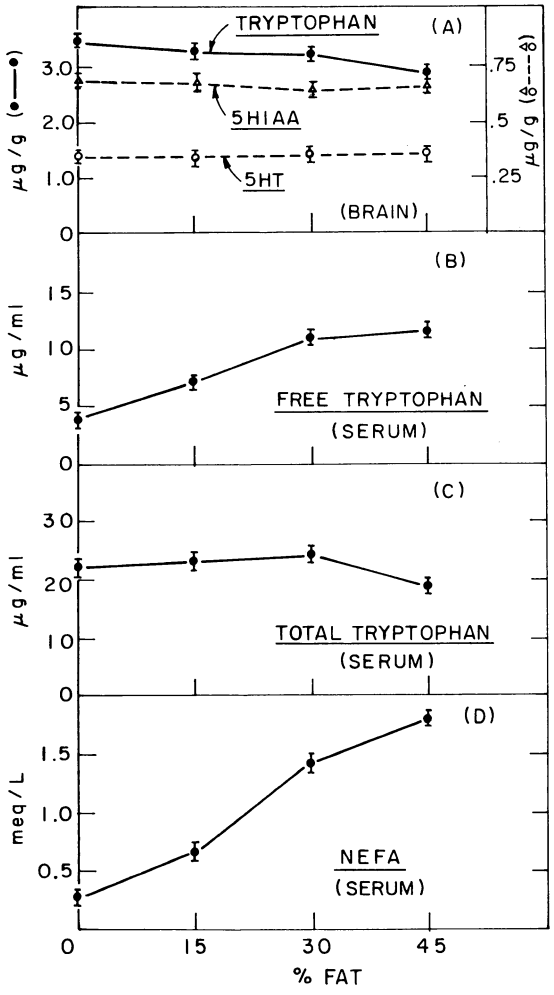

Fig. 1. Effect of dietary fat on serum free tryptophan and brain indoles. Groups of animals (12 per group) were presented with one of four synthetic diets. These were: (1) 0% fat: casein (200 g), sucrose (203 g), dextrose (254 g), dextrine (254 g); (2) 15% fat: mazola oil (150 g), casein (200 g), sucrose (160 g), dextrose (200 g), dextrine (200 g); (3) 30% fat: mazola oil (300 g), casein (200 g), sucrose (116 g), dextrose (147 g), dextrine (147 g); and (4) 45% fat: mazola oil (450 g), casein (200 g), sucrose (74 g), dextrose (93 g), dextrine (93 g). Each diet was supplemented with choline chloride (2 g/4 ml), vitamin mix (10 g), and Rogers-Harper's salt mix (40 g) and suspended in an agar base (35 g/liter H_2O). The average hourly food consumptions by the four groups were 0%, 5 g per rat; 15%, 8 per rat; 30%, 10 g per rat; and 45%, 8 g per rat. Rats were killed 2 hr after presentation of food.

Table 2. Lack of Correlation Between Diet-induced Increases in Serum Unbound Tryptophan, With Tissue Tryptophan Concentrations

	Fat Content of Diet			
	0%	15%	30%	45%
Serum unbound tryptophan μg/ml	4.1 ± 0.2	7.1 ± 0.6*	11.7 ± 1.0*	12.0 ± 0.7*
Muscle tryptophan μg/g	10.8 ± 0.7	12.3 ± 0.7†	11.7 ± 0.9†	10.3 ± 0.5†
Small intestine tryptophan μg/g	29.6 ± 2.2	27.7 ± 1.6†	24.6 ± 1.0†	27.2 ± 1.3†
Liver tryptophan μg/g	24.1 ± 1.7	22.3 ± 2.0†	26.5 ± 1.5†	23.7 ± 1.7†

Rats were treated as described in Fig. 1.

*$p < 0.001$.

†N.S.

ing the 45% fat diet (Fig. 1). The concentration of unbound tryptophan in the serum increased as a function of dietary fat content between 0% and 30% fat, but not thereafter. Even though serum unbound tryptophan was 75% higher in animals consuming the 15% fat meal than in those eating the nonfat meal, and 185% and 192% higher in the 30% and 45% fat groups, none of the fat-containing diets caused brain tryptophan, serotonin, or 5-hydroxyindoleacetic acid to rise beyond the concentrations observed in the nonfat group (Fig. 1). The almost twofold increases in serum unbound tryptophan observed in the animals

Table 3. Effect of Prolonged Consumption of a High-fat Diet on Serum Unbound Tryptophan and Tissue Tryptophan

	Fat Content	
	5%	30%
Initial body weight (g)	202 ± 5	208 ± 4
Final body weight (g)	228 ± 4	231 ± 4
Serum total tryptophan μg/ml	24.6 ± 1.2	25.0 ± 0.8
Serum unbound tryptophan μg/ml	4.9 ± 0.2	5.3 ± 0.3
Serum NEFA meq/liter	0.385 ± 0.053	0.406 ± 0.016
Liver tryptophan μg/g	18.9 ± 0.8	24.1 ± 1.6*
Small intestine tryptophan μg/g	32.4 ± 1.3	29.9 ± 1.5
Brain tryptophan μg/g	5.6 ± 0.2	6.1 ± 0.3
Muscle tryptophan μg/g	8.2 ± 0.3	7.8 ± 0.3

Two groups of rats (10 rats per group) were allowed to eat a mixed meal containing either 5% fat or 30% fat ad-lib. for 3 days. They were killed at noon or at least 6 hr after the cessation of food consumption. Animals eating the high-fat meal consumed 24, 31, 31 g/rat (average) of food on the 3 days studied; those eating the low-fat diet consumed 24, 41, 42 g/rat (average), respectively.

*$p < 0.05$, differs from group consuming 5% fat diet.

eating 30% or 45% fat diets were also unassociated with any significant changes in the tryptophan concentrations of striated or intestinal smooth muscle (Table 2), or in hepatic tryptophan pyrrolase activity (data not included).

Effect of Prolonged Consumption of Dietary Fat on Serum Unbound Tryptophan

To determine whether the repeated consumption of a high-fat diet affects tissue tryptophan levels, we gave animals access ad-lib. to diets containing 5% or 30% fat for 3 days; they were killed at noon. Both groups of animals gained equivalent amounts of weight during the experimental period (Table 3). Serum unbound tryptophan, total tryptophan, and NEFA concentrations did not differ significantly between the experimental groups, probably because the animals had not eaten for at least the previous 6 hr, nor did the tryptophan concentrations of the brain, small intestine, or sketetal muscle differ. Hepatic tryptophan concentrations were 34% greater ($p < 0.5$) in the rats that had eaten the 30% fat diet. These data show that dietary fat level does not permanently affect NEFA concentration or, consequently, the proportion of unbound tryptophan in the serum. The changes observed in the preceding experiments are thus confined to the absorptive state.

Comparison of Different Procedures for Measuring Bound and Unbound Tryptophan

Published values for the absolute concentrations of unbound tryptophan in serum and plasma vary considerably,[1-4,6-8,18,19] possibly because different methods have been used to separate the free from the albumin-bound amino acid. To determine whether our failure to observe correlations between diet-induced changes in serum unbound tryptophan and tissue tryptophan concentrations resulted from artifacts introduced by the method that we used for measuring the unbound tryptophan, we repeated our experiments using three of the commonly used published methods for separating and assaying unbound tryptophan. Sera from rats given access to no-fat or high-fat (30%) diets (prepared as described in Fig. 1) were assayed for unbound tryptophan after equilibrium dialysis at 37°C (i.e., our standard method), after equilibrium dialysis at 0°C (a temperature at which the probability of liberation of NEFA by serum lipase would be diminished), and after ultrafiltration at 25°C.

Regardless of the method employed, the consumption of a high-fat diet caused serum unbound tryptophan concentrations to be at least double those observed in animals eating the fat-free diet (Table 4). The unbound tryptophan concentration of serum was highest (5.6 μg/ml) when measured in low-fat samples obtained by equilibrium dialysis at 37°C and at pH 7.5. In samples dialyzed at 0°C, but also held at pH 7.5, free tryptophan levels were lower than those obtained at 37°C, probably because the binding affinity is greater at reduced temperatures.[20] Unbound tryptophan levels were lowest (2.6 mg/ml) in samples prepared by ultrafiltration, during which the pH was unregulated, reaching values of 8 or more; thus, the reduced temperature and high pH both enhanced the binding of tryptophan[20] during ultrafiltration. To determine whether unbound tryptophan concentrations in samples prepared by dialysis at 37°C were

Table 4. Measurement of Free Tryptophan Separated From Serum Albumin-bound Tryptophan by Three Methods

	0% Fat	30% Fat
Serum total tryptophan μg/ml	31.9 ± 1.0	32.9 ± 1.5
Serum free tryptophan equilibrium dialysis at 37°C for 3.5 hr	5.6 ± 0.3 (17.6% of total)	14.4 ± 1.2 (43.8% of total)
Serum free tryptophan equilibrium dialysis at 0°C for 24 hr	2.9 ± 0.1 (9.1% of total)	12.9 ± 1.6 (39.2% of total)
Serum free tryptophan ultrafiltration 800 g, 30 min ambient temperature	2.6 ± 0.4 (8.2% of total)	5.2 ± 0.5 (15.8% of total)
Serum NEFA meq/liter before dialysis	0.239 ± 0.023	1.009 ± 0.101
Serum NEFA meq/liter after dialysis at 37°C for 3.5 hr	0.236 ± 0.020	1.091 ± 0.085
Serum NEFA meq/liter after dialysis at 0°C for 24 hr	0.228 ± 0.023	0.931 ± 0.060

Groups of 35 animals were fasted overnight and presented with food containing either 0% fat or 30% fat, prepared as described in Fig. 1. The animals were allowed to eat for 2 hr; then individual rats in each group were killed alternately. Serum samples from four animals were pooled, and trytophan was assayed as described in the text.

artifactually high because serum lipase had liberated additional NEFA during the incubation, we measured NEFA levels before and after dialysis at 0°C and 37°C in serum samples obtained from rats consuming high- or low-fat diets (Table 4). The NEFA content of the high-fat samples incubated at 37°C increased by 10% during the dialysis period, but this change was not significant; the NEFA content of the samples incubated at 0°C did not change. Dialysis did not alter the NEFA levels in the low-fat samples.

DISCUSSION

The present observations confirm[2] that the concentrations of free tryptophan in the serum, and of tryptophan in the brain, do not always change in parallel. In studies from other laboratories in which parallel changes were observed, the experimental manipulations used to modify tryptophan levels included starvation,[7,8] immobilization,[7] treatment with drugs that bind to albumin,[6] or injections of large amounts of tryptophan.[8] Our finding that major variations in serum free tryptophan (caused by the elective consumption of diets containing various proportions of fat, Fig. 1) can be unaccompanied by any changes in brain tryptophan suggests that the apparent correlation between brain and serum free tryptophan noted after these other treatments may have been fortuitous or might occur only when there are massive, unphysiologic shifts in tryptophan binding. (It is possible that the flux of unbound tryptophan into the brain and its subsequent turnover were accelerated in our studies when the size of the unbound pool increased; however, this seems unlikely since brain sero-

tonin and 5-hydroxyindoleacetic acid concentrations also failed to exhibit any correlations with serum free tryptophan [Fig. 1].)

The interaction of tryptophan with albumin is a reversible process, and the free and bound pools are in an equilibrium that obeys the law of mass action. If the affinity of brain transport sites for tryptophan is greater than the affinity of albumin for the amino acid, this condition would enable the brain to withdraw tryptophan from not only the free pool in plasma, but also, by mass action, from the bound pool. A similar process appears to regulate the flux of plasma NEFA into certain tissues, inasmuch as their tendency to be transported in blood almost entirely bound to albumin (> 99%) does not prevent them from being rapidly transported into tissues and/or from having a very short (1–2 min) half-life in plasma.[12]

The elevation in brain tryptophan levels following carbohydrate ingestion confirms previous findings[9] and provides further indirect evidence that brain tryptophan levels are regulated by the ratio of tryptophan to neutral amino acids which compete with tryptophan for entry into the brain.[10] Thus, after carbohydrate is eaten and insulin secreted, tryptophan levels in serum or plasma are stable or rise[2,9,22,23] at a time when concentrations of other neutral amino acids rapidly fall.[22,24] Plasma tryptophan responds to insulin differently from the other neutrals because the amino acid is bound to albumin. After a carbohydrate meal NEFA levels fall; this enhances the binding of tryptophan to albumin, raising the concentration of albumin-bound tryptophan and precluding a decline in total serum tryptophan (Table 1). The increase in the ratio of serum tryptophan to other neutral amino acids enhances the competitive position of tryptophan competing for brain uptake; hence, brain tryptophan rises.

The concentrations of tryptophan in liver and intestine did not change after acute diet-induced fluctuations in the serum unbound pool (Tables 1 and 2), suggesting that the magnitude of this pool also does not govern the amount of circulating tryptophan that is available for uptake by these tissues. The activity of liver tryptophan pyrrolase, an enzyme that is induced by its amino acid substrate,[17] failed to respond to physiologic variations in serum unbound tryptophan. In contrast, Badawy and Smith observed an increase in tryptophan pyrrolase activity 3 hr after serum tryptophan was displaced from albumin binding sites by salicylates.[18] This increase was observed only when the animals received tryptophan in addition to the drug. Chronic administration of antirheumatic drugs, including salicylates, to humans reportedly reduces serum total tryptophan concentration and increases the free component by dislodging tryptophan from albumin.[19] In our studies, the consumption for 3 days of a high-fat diet which acutely elevates serum free tryptophan (Fig. 1) had, in the postabsorptive state, no continuing effect on serum total or unbound tryptophan, or on tissue tryptophan in rats, except in the liver (Table 3). Thus the effect of NEFA on tryptophan binding is transient and parallels the changes in serum NEFA levels.

The ability of albumin to bind circulating tryptophan may *indirectly* determine the availability of this amino acid to the brain and perhaps to other tissues. The interaction between NEFA and tryptophan on the albumin molecule serves to buffer serum tryptophan and to prevent its concentration from

falling markedly after insulin is secreted (i.e., in contrast with other neutral amino acids). This change in the plasma amino acid pattern makes it possible for the consumption of a protein-poor meal to be followed by an elevation in brain tryptophan and thus to cause an acceleration of the synthesis of the neurotransmitter serotonin.[9]

The reduction of muscle tryptophan after carbohydrate consumption could indicate that brain tryptophan levels are maintained or elevated at the expense of muscle. The possible redistribution of muscle reserves to the brain could be explained by postulating that the affinity of brain for tryptophan was greater (and that of muscle less) than the affinity of albumin for the amino acid. Further experiments will be needed to determine whether these affinities do in fact differ and whether they respond differently to competing neutral amino acids or insulin.

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