Biochemical and Behavioral Effects of a Commercial "Low Phenylalanine" Diet in Growing Rats* (32637)

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(Introduced by S. P. Bessman)
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For children diagnosed at birth to have phenylketonuria, a therapeutic diet restricted in phenylalanine (Phe) is recommended. This results in a lowering of blood Phe (1, 2) which is the only parameter used in pediatric practice for the follow-up of these patients. The nutritional drawback of such a diet is evident: In order to control the phenylalaninemia, the diet must be grossly deficient in Phe. Some phenylketonuric children maintained on Phe-restricted diets have had skin lesions, refractory anemia, bone changes, failure to gain weight, eczema, and listlessness (3, 4). Animal data on the effect of a commercial low Phe diet are very scarce (5), yet are much needed as they might reveal possible dangers.

This study reports physiological, biochemical, and behavioral changes as related to intake of a commercial low Phe diet by young rats. Two enzymes were selected to monitor biochemical changes: phenylalanine hydroxylase (PheH) activity of liver (6) and glutamine synthetase (GS) activity of brain (7). The former was chosen because of its importance in the first step of Phe breakdown. Since the latter enzyme is linked with the metabolism of amino acids by removing endogenous ammonia in the form of glutamine and thus preventing toxic effects to the brain (8), it could be used as a biochemical index of brain metabolism changes and furthermore might be related to behavioral variations in the experimental animals.

Materials and Methods. Pregnant albino Wistar rats were purchased in the third week of gestation weighing approximately 300 gm. They were placed in individual cages and fed Purina Laboratory Chow (PuLabChow) until delivery. The size of the litter was adjusted to no more than 10. If a litter contained more than 10 pups, the surplus was removed and, if necessary, transferred to a litter containing less than 10, provided the difference in age was no more than 12 hours. Starting immediately after delivery, pairs of mother rats were placed on the same experimental diet for the following 3 weeks. The pups were then separated from the mother. Two pups from every litter were used for biochemical determinations and the remaining were divided into two groups which were kept two rats per cage, and fed an experimental diet for the next 5 weeks. Food intake and weight gain were recorded daily. For biochemical determinations, rats from each parallel group were killed at age 28, 42, and 56 days. The animals were anesthetized lightly with ether, then decapitated, and the blood was collected in centrifuge tubes. Behavioral testing was done at 45 and 52 days of age.

The basic diet was Lofenalac[2] (LoPhe). It was given *ad libitum* as a 15% solution, as recommended by the the manufacturers,

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[1] Present address: Food and Drug Administration, Washington, D.C.

[2] Mead Johnson Laboratories, Evansville 21, Ind. The chemical composition of 100 gm of LoPhe powder is stated to be as follows: total nitrogen (equiv. to 15% protein), 2.4 gm; fat, 18 gm; carbohydrate, 57 gm; minerals, (ash) 5 gm (calcium 0.65%, phosphorus 0.5%, iron 0.01%); moisture, 2 gm; isoleucine, 0.75 gm; leucine, 1.41 gm; lysine, 1.57 gm; methionine, 0.51 gm; phenylalanine, 0.08 gm; threonine, 0.77 gm; tryptophan, 0.19 gm; valine, 1.20 gm; arginine, 0.34 gm; histidine, 0.26 gm; alanine, 0.64 gm; aspartic acid, 1.34 gm; cystine, 0.025 gm; glutamic acid, 3.78 gm; glycine, 0.35 gm; proline, 1.13 gm; serine, 1.02 gm; tyrosine, 0.81 gm; vitamin A palmitate, 690 units; vitamin D (calciferol), 183 units; vitamin E, 2.3 units; ascorbic acid, 13.7 mg; thiamine hydrochloride, 0.21 mg; riboflavin, 0.83 mg; niacinamide, 1.83 mg; pyridoxine hydrochloride, 0.23 mg; calcium panthotenate, 1.47 mg; vitamin B_{12}, 2.07 mg; folic acid, 0.02 mg; biotin, 0.014 mg; choline chloride, 69 mg.

with additional L-Phe[3] of 0, 0.2, 0.4, 0.8 and 5.0% concentration. The L-Phe was first dissolved in hot water, cooled and added to the LoPhe powder in a Waring blender. PuLab-Chow containing 0.8% L-Phe and approximately 0.3% L-tyrosine (Tyr) was used as a solid diet control.

Phe and Tyr were determined in serum by micromethods (9, 10). PheH activity was measured in the liver (11, 12). Results are expressed as μmoles $\times$ 10^{-4} of Tyr formed per milligram of protein in 30 min at 25°C. Total protein was determined in all tissue homogenates and supernatants by a spectrophotometric micromethod (13).

The GS activity was measured in the brain. For this the brain was homogenized in 3 volumes of freshly prepared 0.25 *M* sucrose containing 0.6% sodium deoxycholate. This was followed by a 90-min dialysis at 0°C against water. Elliott's assay (14) for GS was used except for the following modifications: to 2.0 ml of incubation mixture, 0.25 ml of dialyzed brain homogenate was added. Incubation was carried out in duplicate for 10 min at 37°C in a Dubnoff shaking water bath, after which time the reaction was stopped with 0.75 ml of ferric chloride reagent (5 parts 20% $FeCl_3 \cdot 6\ H_2O$ in 0.2 *N* HCl, 3 parts 30% trichloroacetic acid, 1 part concentrated HCl). This particular reagent (15) was found to produce better stability of color than the one originally recommended (14). The precipitated proteins were centrifuged and the supernatant was read within 45 min in a Klett spectrophotometer at 540 mμ against water. Zero time controls were carried out simultaneously. A standard curve was established according to the assay procedure above with glutamohydroxamic acid (GHA) in lieu of enzyme solution. The GHA was prepared according to Lipmann & Tuttle (16). Results are expressed as specific activity, *e.g.*, micromoles of GHA synthesized per milligram of protein in 10 min at 37°C.

Behavioral testing consisted of a 30-min session in an operant conditioning Lehigh Valley test chamber which had been programmed for a Sidman avoidance schedule of response–shock interval of 10 sec (RS-10) and a shock–shock interval of 5 sec (SS-5). Under this schedule, each time the animal presses the lever the 0.5 mA shock is delayed by 10 sec (17). The total number of responses (total output), shocks and the number of responses occurring in the RS intervals were automatically recorded. The results are expressed as total output, percentage of avoidance and percentage of RS responses.

Results. We had planned originally to place mother rats at the time of delivery on LoPhe and at weaning time to continue the offspring on this very same diet. However, it soon became apparent that mother rats on LoPhe could not raise a viable litter. A deficient lactation, possibly due to a decrease of milk production or the complete lack of it, leading to starvation of the pups was observed. Within 2 weeks, they either died or were eaten by their mothers. In order to assure viable offspring, mother rats had to be given at least 0.2% additional L-Phe during the 3 weeks after delivery. At weaning time half the offspring were continued on the same diet as the mother while the other half were placed on LoPhe for the following 5 weeks.

Table I summarizes the enzymatic findings from the groups of young rats placed on LoPhe. The amount of L-Phe in the diet of the mother is directly related to the brain GS and indirectly to the liver PheH of the offspring at weaning. Withdrawal of L-Phe did not produce any drop in GS. The increase in liver PheH, however, was temporary: at 56 days much less enzyme was found than normally measured at this age (Table II). Physical growth of these rats was almost immediately arrested when dietary Phe was restricted and the weight gain over the next 5 weeks was negligible (Fig. 1). Mortality among the rats was high. Survivors were hyperactive at times and dull at others. Their fur became thin and had a yellow tinge. The overall physical condition of these animals was poor.

When 0.2% L-Phe was added to the diet, liver PheH was found to be higher than normal at 21 days (Table II), the increased value was maintained for a week and then fell. At the same time serum Phe and Tyr remained within normal limits (Fig. 2).

When L-Phe was increased to 0.4% or more (Table II), the starting value for PheH was

[3] Sigma Chemical Co., St. Louis 18, Mo.

not as pronounced anymore. Addition of 0.8% or 5.0% L-Phe did not prevent the enzyme from decreasing gradually; at 56 days all rats on any type of liquid diet had liver enzyme of less than half the value of control rats on a solid diet. At the same time all Phe serum levels were identical regardless of the amount of dietary Phe or of the consistency of the diet (Fig. 2). A diet with 5.0% L-Phe was insufficient to produce hyperphenylalaninemia.

The GS in the mothers' brains was not affected by the amount of L-Phe provided in the diet (Table III): all GS activities were within the range of the controls. However, the more deficient diets resulted in decreased brain GS activity in the offspring at weaning (Tables I and IV). The decrease was temporary only, as the enzyme activity returned to normal within a week. We have observed that rats growing on a solid control diet nor-

TABLE I. Brain Glutamine Synthetase (GS) and Liver Phenylalanine Hydroxylase (PheH) of Young Rats Raised on a LoPhe Diet.

	Diet of mother rat during lactation					
	LoPhe	LoPhe + 0.2% L-Phe		LoPhe + 0.4% L-Phe		LoPhe + 0.8% L-Phe	
Age (days)		GS	PheH	GS	PheH	GS	PheH
21[a]	No survivors	1.08	42.3	1.11	31.0	1.43	31.7
		Offspring continued on LoPhe					
28		1.50	47.5	1.38	38.0	1.38	18.5
42		1.35	56.6	1.30	30.0	1.35	—
56		1.26	23.5	Died		1.50	13.0

[a] Rats pooled. Results obtained at age 28, 42, and 56 days are the average of two rats of the same litter.

TABLE II. Phenylalanine Hydroxylase Activity in Liver Homogenates.

	Diet[a]				
Age (days)	LoPhe + 0.2% L-Phe	LoPhe + 0.4% L-Phe	LoPhe + 0.8% L-Phe	LoPhe + 5.0% L-Phe	PuLabChow
21	(2) 42.3	(4) 25.5 ± 6.4	(2) 31.9	(2) 31.7	(6) 21.7 ± 2.1
28	(4) 46.5 ± 7.5	(2) 19.0	(2) 21.0	(2) 29.2	(4) 40.7 ±15.6
42	(3) 18.3 ±11.9[b]	(2) 30.0	(6) 12.5 ± 6.6[b]	(4) 27.2 ± 11.4[b]	(8) 46.1 ± 5.7
56	(6) 19.0 ± 9.3[b]	(4) 21.9 ± 0.2[b]	(8) 20.3 ± 5.5[b]	(4) 20.7 ± 6.9[b]	(8) 52.5 ± 7.7

[a] The number in parenthesis is the number of animals used for every experimental stage. Average values plus or minus the standard deviation.

[b] $p < .05$ compared to the Purina Laboratory Chow animals of the same age.

TABLE III. Effect of Diet on Brain Glutamine Synthetase of Lactating Mother Rats. (Specific activity determination done on day of weaning)

	Diet				
LoPhe	LoPhe + 0.2% L-Phe	LoPhe + 0.4% L-Phe	LoPhe + 0.8% L-Phe	LoPhe + 5.0% L-Phe	PuLabChow
1.34	1.50	1.29	1.29	1.32	1.34 ± 0.11[a]

[a] Average of 10 rats; all other results are the average of two.

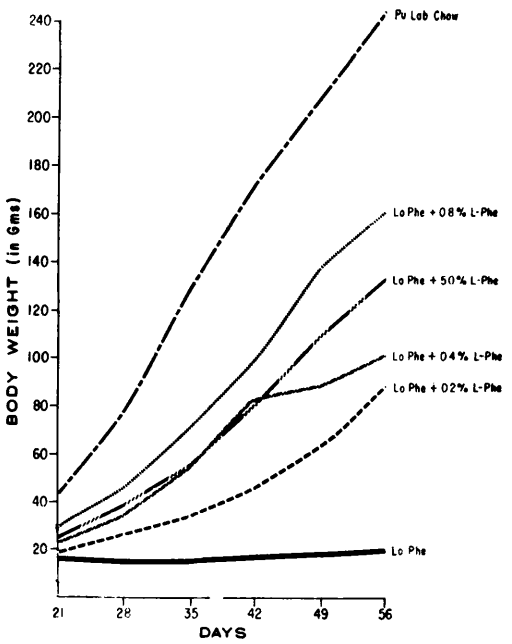

FIG. 1. Growth curves of rats fed diets containing various amounts of L-phenylalanine (L-Phe); LoPhe: Lofenalac containing 0.1% L-Phe; and PuLabChow: Purina laboratory chow containing 0.8% L-Phe.

mally exhibit a peak in brain GS activity within the first week after weaning. This peak was not noted in rats on any type of liquid diet. At 56 days all rats except those on 0.8% and 5.0% L-Phe were well adjusted to their diet and exhibited normal brain GS activities. The 5.0% L-Phe seemed to have a deteriorating effect on the enzyme as there was a rapid initial decrease and no recovery over the entire 5-week period.

Figure 1 shows the growth curve of rats on LoPhe diets with and without additional L-Phe. Growth was virtually arrested in rats on LoPhe, and enhanced as more L-Phe was provided, but even with the established optimum of 0.8% L-Phe (18, 19) there was considerably less growth than was obtained by rats on PuLabChow. The difference in weight was approximately 100 gm.

The animals were tested behaviorally at 45 and 52 days of age, a time shown (20) to be a critical period for the measurement of behavioral deficits. As the minimum necessary weight for testing was 75 gm, all animals weighing less had to be excluded. Rats on LoPhe plus 0.2% L-Phe or less did not attain the minimum testing weight within 45 days. Therefore, only animals from the remaining three experimental groups could be tested. Due to the striking difference in weight between rats on PuLabChow (Control A) and these three groups, it became necessary to introduce another control, e.g., rats on PuLabChow of equivalent weight (Control W) rather than of equivalent age. Rats chosen as Control W had a weight approximately equal to the average weight attained by the three experimental groups at the testing age. Thus, Control W rats are younger than Control A rats.

Control A (Fig. 3) showed a normal record of high responses with more than 40% avoidance of shocks in the first trial and over 60% of the responses in the RS interval. In the second test, the total number of responses and percentage of avoidance were increased, but there was no significant increase in the RS responses. Control W showed a poorer overall performance at the first trial. However, a week

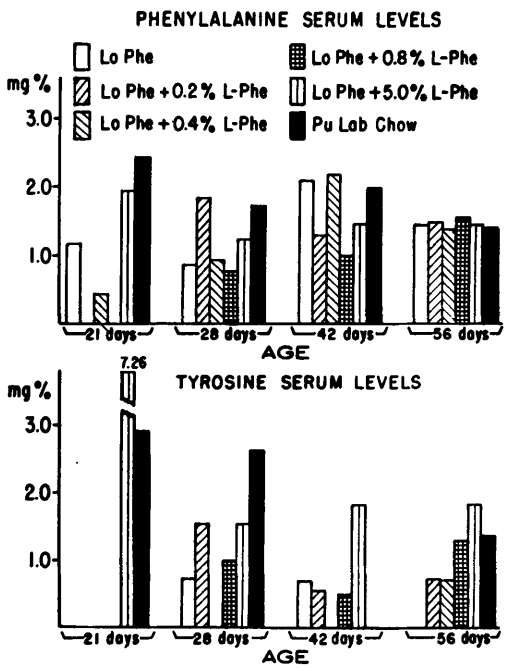

FIG. 2. Phenylalanine and tyrosine serum levels as related to diets containing various amounts of L-phenylalanine (L-Phe); LoPhe: Lofenalac containing 0.1% L-Phe; and PuLabChow: Purina laboratory chow containing 0.8% L-Phe.

TABLE IV. Glutamine Synthetase Activity in Rat Brain Homogenates.

Age (days)	Diet: LoPhe + 0.2% L-Phe	LoPhe + 0.4% L-Phe	LoPhe + 0.8% L-Phe	LoPhe + 5.0% L-Phe	PuLabChow
21	(2) 1.08	(4) 1.11 ± 0.05[a]	(5) 1.42 ± 0.13	(2) 1.47	(6) 1.35 ± 0.05
28	(4) 1.15 ± 0.14[a]	(2) 1.36	(2) 1.28	(2) 1.11	(4) 1.66 ± 0.10
42	(5) 1.17 ± 0.18	(2) 1.28	(3) 1.29 ± 0.24	(4) 1.12 ± 0.05	(4) 1.30 ± 0.04
56	(6) 1.25 ± 0.21	(4) 1.45 ± 0.09	(4) 1.11 ± 0.08	(4) 0.93 ± 0.08[a]	(4) 1.33 ± 0.06

[a] $p < .05$ compared to the Purina Laboratory Chow animals of the same age.

later, the increase in the number of RS responses was greater than in the Control A. Rats on LoPhe plus 0.4% L-Phe showed very poor performance in the first trial but improved in the second one. Rats on 5.0% L-Phe diet showed a low total output and percentage of avoidance with a rather high percentage of RS responses in the first trial. However, at the second test, there was a marked decrease in percentage of RS responses and a slight decrease in total output which suggests some behavioral deficit in the retention of the learned responses.

Discussion. Major brain growth of the rat is completed by weaning time when the rat is approximately 3 weeks old. Therefore, any dietary treatment expected to have a damaging effect on brain growth would have to be started as soon as possible after birth and very probably within the first 3 weeks of life. Direct administration of a liquid diet to newborn rats is technically impractical; therefore, we had to resort to placing mother rats on the special diet and observe its indirect effect on the offspring. A diet low in L-Phe affected the brain GS of young rats as shown by enzymatic assays carried out at weaning time. Interestingly enough, although they showed severe weight loss mother rats had no loss of brain GS on the LoPhe diet. The young rats managed to adjust rather quickly to whatever diet they were given in terms of GS but PheH was always low. Physical growth, however, was impaired and the behavioral testing showed signs of immaturity. We did not notice any work in the literature pertaining to the biochemical effects of LoPhe diets, with the exception of the work of Freedland *et al.* (5) who demonstrated a hepatic PheH decrease in 28 to 30-day-old rats after 7 days on LoPhe or after 1–3 days of fasting. We have found the same 50% decrease of the enzyme in adult male rats. Weight loss was approximately 5% for rats on LoPhe and about 10% for those on 48 hours of starvation. These adult male rats were on LoPhe for a total of 3 weeks. Brain GS and liver PheH dropped temporarily after 1 week, then increased to above the con-

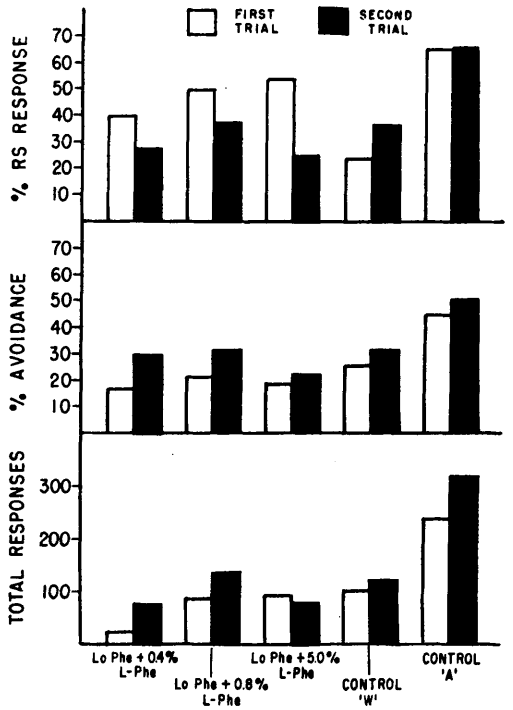

FIG. 3. Results of the Sidman avoidance test. For details of the test see "Materials and Methods." LoPhe: Lofenalac containing 0.1% L-Phe, control 'A': rats (raised on Purina laboratory chow) of the same age as the experimental groups; and control 'W': rats (raised on Purina laboratory chow) of the same weight as the means calculated from all three experimental groups.

trols on PuLabChow before returning to normal. These data suggested increased protein catabolism during the period of adaptation. The same interpretation might be given to the results of GS assays in weanling rats on LoPhe (Table I). Since all rats on LoPhe plus 0.8% L-Phe did not gain as much weight as the controls on solid food and since the rats on liquid diets with less than 0.8% L-Phe gained even less, the question arose as to whether the rats did not get enough nourishment because of their limited stomach capacity and dilution of the nutrients. This problem was also discussed in detail by Shapiro *et al.* (21). We prepared a solid LoPhe diet from equal parts of LoPhe and nonnutritive fiber[4] which was homogenized in the Waring blender with sufficient water to give a thick liquid. It was poured in a flat dish and evaporated at room temperature under a current of air. This dry cake form diet was given *ad libitum* with water separately and was accepted by the weanling rats as readily as the liquid 15% LoPhe solution. The lack of weight gain and the appearance of the rats were similar to that resulting from liquid LoPhe. The same dry cake form diet but containing 0.8% L-Phe was given as a solid control to the liquid 15% LoPhe solution containing 0.8% L-Phe. The rate of growth was equal regardless of the way of preparation of the diet and below that of rats raised on PuLabChow. This again brings up the question whether natural protein is superior to an equivalent amount of hydrolyzed protein (22).

In our study, weaning has been arbitrarily chosen to be day 21, although due to individual difference among litter mates this day may well be shifted in both directions by a few days. It is therefore conceivable that the peak period of brain GS activity following weaning reflects the adjustment of the rat from mother's milk to food of a different chemical composition. The 21 day value in our study is an indication of how the mother's diet affected the young and growing rat indirectly through the mother's milk. Therefore, the lower than normal brain GS found in rats whose mothers were on low Phe diets (LoPhe plus 0.2% or plus 0.4% L-Phe) could be interpreted to mean a lag in maturation which is also indicated by the results of the behavioral testing. The Phe and Tyr serum levels never reflected the Phe intake, and even with 5.0% L-Phe in the diet, no hyperphenylalaninemia could be achieved which is in agreement with Auerbach, Waisman, and Wyckoff (23) and with Boggs and Waisman (24). The finding that 5.0% L-Phe lowered both brain GS and liver PheH makes the assumption likely, that this particular dose of L-Phe inhibits protein synthesis in general. Polidora *et al.* (20) reported that a diet with 5.0% L-Phe started after weaning produced only reversible behavioral changes and not a permanent one. Schalock and Klopfer (25) gave high Phe by tube to rats starting on the first day of life and reported behavioral abnormalities after 30 days, in discrimination and reasoning tests.

Summary. Liquid diets made from LoPhe powder and water and containing additional L-Phe in amounts of 0.2, 0.4, 0.8, and 5.0% were given to mother rats immediately after delivery for the following 3 weeks and then to the offspring for the next 5 weeks. The result was a faulty development of liver PheH and brain GS. An overall impairment of growth and behavior tests done at age 45 and 52 days suggested a lag in maturation of approximately 10 days. LoPhe without additional L-Phe could be fed to rats only after day 21. When given to mother rats from the time of delivery, offspring did not survive. Although highly deficient in L-Phe and producing a complete arrest of growth, this diet resulted in normal brain GS of offspring; however, liver PheH was greatly decreased after a temporary rise. The 5.0% L-Phe produced neither phenylalaninemia nor an adaptation of PheH, but decreased brain GS slowly and consistently over the entire time of the study. The Phe and Tyr serum levels at no time correlated with the dietary intake of these two amino acids. Our data show, at least for the rat, that serum Phe levels in growing animals do not reflect adequacy of intake of Phe.

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Inhibition of the Release of Slow-Reacting Substance of Anaphylaxis in the Rat with Diethylcarbamazine* (32638)

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Diethylcarbamazine citrate (Hetrazan) is an effective chemotherapeutic agent against microfilarial infestations in animals and man (1); however, the mode of action of this piperazine derivative on microfilariae remains to be established. Hetrazan has also been utilized with success in the treatment of tropical eosinophilia when no microfilarial infestation could be demonstrated (2). More recently, Mallén noted the efficacy of this drug in the relief of the bronchospastic symptoms associated with tropical eosinophilia, and this observation prompted him to perform a brief clinical trial with Hetrazan in "intractable asthma" (3). Fourteen of 15 patients responded satisfactorily to Hetrazan with only minimal side effects.

Slow-reacting substance of anaphylaxis (SRS-A) has been suggested as a chemical mediator involved in the pathogenesis of human asthma (4). Brocklehurst (5) has demonstrated the release of SRS-A_{hu} from the perfused segments of lung of 2 pollen-sensitive individuals and from a bronchial ring preparation which contracted in response to specific antigen. In addition, human bronchiolar tissue is the only bronchiolar tissue known to be very sensitive to guinea pig SRS-A_{gp} (4).

The immunologic release of SRS-A_{rat} has been accomplished *in vivo* in the rat using both heterologous (6) and homologous (7) antisera. The SRS-A_{rat} recovered from the rat peritoneal cavity was pharmacologically in-

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