thawed embryos transferred developed into either dark-eyed living fetuses or newborn mice that appeared entirely normal. None appeared abnormal. In the 13 animals allowed to litter, 48 percent of 120 transferred embryos developed into black or agouti pups. (These mice have yielded normal litters, both when mated with each other and with a normal control mouse). In the 64 females examined before term, 42 percent of the transferred embryos had developed into fetuses and 23 percent more had implanted but had been resorbed. Thus 65 percent of the 501 embryos in pregnant females underwent implantation. Fetuses and live-born mice developed normally from embryos cooled to −78°, −196°, or −269°C, regardless of whether transfer was immediately after thawing or after development to blastocysts. Since 43 percent of the embryos in pregnant females underwent complete development and since the frozen-thawed embryos had been pooled before transfer, it is statistically unlikely that any animal failed to become pregnant because the six to ten embryos it received were all nonviable. Rather, the nonpregnancy was probably a physiological failure of the foster mother.

The results in Table 1 compare favorably with those obtained for the transfer of normal, unfrozen embryos into mice (*21*). Thus, embryos that survive freezing and thawing (with capacity to develop to blastocyst in culture as the criterion of survival) are capable of developing to term and producing normal, living young. Our results imply (*2*) that storage of mutant strains of mice not in current use but of potential interest is now possible. As needed, the stored embryos could be thawed and transferred to foster mothers, and the resulting offspring used to reestablish the mutant strain. If these procedures prove applicable to embryos of large domestic animals, they would also facilitate worldwide dissemination of stock with an optimal genetic background for a particular use or geography. Finally, the success of cryobiological theory in suggesting the proper approach to the freezing of these sensitive embryos increases the likelihood that ways can be found to freeze complex mammalian systems for medical use.

D. G. WHITTINGHAM*
S. P. LEIBO, P. MAZUR
Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

References and Notes

1. J. K. Sherman, *Cryobiology* **1**, 103 (1964).
2. D. G. Whittingham, *Nature* **233**, 125 (1971).
3. J. D. Biggers, W. K. Whitten, D. G. Whittingham, in *Methods of Mammalian Embryology*, J. C. Daniel, Jr., Ed. (Freeman, San Francisco, 1971), pp. 86–116.
4. D. G. Whittingham and R. G. Wales, *Aust. J. Biol. Sci.* **22**, 1065 (1969).
5. S. P. Leibo, J. Farrant, P. Mazur, M. G. Hanna, Jr., L. H. Smith, *Cryobiology* **6**, 315 (1970).
6. D. G. Whittingham, *J. Reprod. Fert.*, Suppl. 14, 7 (1971).
7. R. L. Brinster, *Exp. Cell Res.* **32**, 205 (1963).
8. Survival of one-cell embryos was based on the fraction developing into two-cell embryos in culture. Further development of experimentals and controls was blocked by the high (20 percent) oxygen in the incubator; however, such arrested embryos have been shown previously to continue development when transferred to foster mothers [D. G. Whittingham, *J. Cell Biol.* **31**, 123A (1966); ——— and J. D. Biggers, *Nature* **213**, 942 (1967)].
9. A. K. Tarkowski, *Acta Theriol.* **2**, 251 (1959).
9a. Embryos were also frozen in 7.5 percent polyvinylpyrrolidone to −78°C at rates from 1° to 600°C min^{-1}, but none survived. Time did not permit us to determine the factors responsible for the difference between these results and successful results reported previously (*2*).
10. P. Mazur, *Science* **168**, 939 (1970).
11. ———, *J. Gen. Physiol.* **47**, 347 (1963); *Cryobiology* **2**, 181 (1966).
12. H. T. Meryman, Ed., *Cryobiology* (Academic Press, New York, 1966).
13. Pooled mean survivals of samples cooled at 0.4, 0.9, 1.2, and 1.9°C min^{-1}. Unless noted otherwise, all other data in this report involved controlled cooling to −110°C, transfer to −196°C, and warming at 4 to 5°C min^{-1} (usually) or 25°C min^{-1} (occasionally).
14. J. K. Sherman, *J. Cell. Comp. Physiol.* **61**, 67 (1963).
15. P. Mazur, S. P. Leibo, J. Farrant, E. H. Y. Chu, M. G. Hanna, Jr., L. H. Smith, in *The Frozen Cell* (Ciba Foundation Symposium), G. E. W. Wolstenholme and M. O'Conner, Eds. (Churchill, London, 1970), pp. 69–85.
16. P. Mazur, S. P. Leibo, R. H. Miller, *Cryobiology* **8**, 383 (1971).
17. J. Farrant reported at the International Congress of Cryosurgery, June 1972, that the optimum cooling rate for certain classes of lymphocytes is about 0.3°C min^{-1}.
18. D. H. Rasmussen and A. P. MacKenzie, *Nature* **220**, 1315 (1968).
19. J. D. Biggers, B. D. Moore, D. G. Whittingham, *ibid.* **206**, 734 (1965).
20. The frozen-thawed embryos had the pedigree Swiss-Webster ♀ × (C57BL × C3H) F_1 ♂ or —more often—(C57BL × C3H) F_1 ♀ × (C57BL × C3H) F_1 ♂.
21. R. J. Mullen and S. C. Carter, *Biol. Reprod.*, in press.
22. Research supported by the Atomic Energy Commission under contract with the Union Carbide Corporation. D.G.W. is a Beit Memorial Fellow and received additional support during his stay from the Atomic Energy Commission and the Medical Research Council of the United Kingdom. We thank H. Long, Thermonuclear Division, Oak Ridge National Laboratory, for making liquid helium facilities available.
* On leave from Physiological Laboratory, Cambridge University, Cambridge, England.

31 July 1972

Brain Serotonin Content: Physiological Regulation by Plasma Neutral Amino Acids

Abstract. *When plasma tryptophan is elevated by the injection of tryptophan or insulin, or by the consumption of carbohydrates, brain tryptophan and serotonin also rise; however, when even larger elevations of plasma tryptophan are produced by the ingestion of protein-containing diets, brain tryptophan and serotonin do not change. The main determinant of brain tryptophan and serotonin concentrations does not appear to be plasma tryptophan alone, but the ratio of this amino acid to other plasma neutral amino acids (that is, tyrosine, phenylalanine, leucine, isoleucine, and valine) that compete with it for uptake into the brain.*

We have shown that when plasma tryptophan concentrations rise in rats receiving low doses of tryptophan (*1*) or subconvulsive doses of insulin (*2*), or in rats consuming a carbohydrate diet (*3*), brain tryptophan and serotonin concentrations also increase. Such variations in amine concentration reflect the general dependence of the rate of serotonin formation on the degree of saturation of tryptophan hydroxylase, the enzyme that catalyzes the rate-limiting step in serotonin biosynthesis (*4*). We suggested that the brain tryptophan elevations were direct responses to the increases in plasma tryptophan, and that, generally, any perturbations which increased plasma tryptophan would similarly increase brain tryptophan and serotonin (*3*). Since dietary protein should elevate plasma trytophan both by eliciting insulin secretion and by providing new tryptophan, we anticipated (*3*) that its consumption should also elevate brain tryptophan and serotonin.

We now report that the elective consumption of protein-containing diets may or may not be followed by increases in brain tryptophan and 5-hydroxyindoles, and that this effect of food consumption on the brain is best correlated not with plasma tryptophan concentration, per se, but with the ratio of plasma tryptophan to five other neutral amino acids that presumably compete with it for uptake into the brain.

Male Sprague-Dawley rats (Charles River Breeding Laboratories) were housed as described in (*3*). At 9 p.m. the evening before an experiment, the

rats were placed in clean cages and deprived of food. Between noon and 3 p.m. the next day, groups of six to eight animals were given free access to one of the following diets; (i) diet 1, a carbohydrate diet (*3*); (ii) diet 2, diet 1 supplemented with 18 percent casein, dry weight; (iii) diet 3, diet 1 supplemented with an artificial amino acid mixture similar to casein in amino acid content (*5*), 18 percent dry weight; (iv) diet 3, but lacking specific amino acids as described below. In all experiments, animals consumed approximately 5 to 7 g of food during the first hour and 3 to 5 g during the second hour. Control rats were fasted and were killed at the beginning of the first hour of the experiment (0-hour control), or 1 or 2 hours later (1-hour and 2-hour controls, respectively). Experimental rats were killed 1 or 2 hours after diet presentation. Blood and brains were collected and prepared as described (*3*). Tryptophan (*6*), serotonin (*7*), and 5-hydroxyindoleacetic acid (*8*) were assayed fluorimetrically. Other plasma amino acids were measured by means of a Beckman model 121 amino acid autoanalyzer.

If rats ate diet 2 (18 percent casein), plasma tryptophan concentrations increased 60 percent above those of fasted controls in 2 hours (0-hour control, 11.44 μg/ml; 2-hour control, 10.46 μg/ml; 2-hour casein, 16.44 μg/ml; $P < .001$); however, brain tryptophan concentrations did not increase (0-hour control, 4.08 μg/g; 2-hour control, 5.07 μg/g; 2-hour casein, 3.47 μg/g). Brain serotonin also failed to rise (0-hour control, 0.58 μg/g; 2-hour control, 0.53 μg/g; 2-hour casein, 0.53 μg/g). The consumption of a standard rat chow (Big Red Laboratory Animal Chow, 24 percent protein, dry weight) produced similar results, that is, a 70 percent increase in plasma tryptophan ($P < .001$) after 2 hours, but no elevations in brain tryptophan or serotonin.

Other investigators, using brain slices (*9*) or animals treated with pharmacologic doses of individual amino acids (*10*), have shown that groups of amino acids (for example, neutral, acidic, basic) are transported into brain by different carrier systems, and that within a given group, the member amino acids compete with each other for common transport sites. Since protein ingestion introduces variable amounts of all amino acids into the blood, brain tryptophan could fail to increase after protein ingestion because the plasma concentrations of other competitor amino acids increase more than does the concentration of tryptophan. To test this hypothesis, we allowed groups of six animals to eat either diet 3 (carbohydrate diet containing the complete amino acid mixture) or diet 3 minus five of the amino acids thought to share a common brain transport system with trytophan (tyrosine, phenylalanine, leucine, isoleucine, and valine). Both diets significantly increased plasma tryptophan above concentrations in fasted control rats (Fig. 1). The plasma concentrations of most other amino acids measured (for example, serine, proline, threonine, and alanine) also increased, except for those omitted from the diet. However, only when the competing neutral amino acids were deleted from the diet did large increases occur in brain tryptophan, serotonin, or 5-hydroxy-indoleacetic acid (Fig. 1).

To rule out the possibility that the differences between rats consuming diet 3 and this diet minus the five competitor amino acids were simply nonspecific effects resulting from the lack of any group of amino acids, we repeated the above experiment omitting aspartate and glutamate from the complete amino acid mixture. These two amino acids comprise approximately the same percent of the total alpha-amino nitrogen in casein as the five competing amino acids. Because they are charged at physiologic *p*H, they are transported into the brain by a different carrier system from that transporting tryptophan (*9*). Hence, their absence should not alter the postprandial competition for brain uptake between tryptophan and other amino acids within its transport group. At 1 and 2 hours after presentation of this diet or of diet 3 (complete amino acid mixture), plasma tryptophan concentrations again increased 70 to 80 percent above those of fasted controls ($P < .001$). However, the ingestion of either diet resulted in no increases in brain tryptophan, serotonin, or 5-hydroxyindoleacetic acid.

These results show that brain tryptophan and 5-hydroxyindole concentrations are not simply a reflection of plasma tryptophan, but also of the plasma concentrations of other neutral amino acids. To illustrate this relation, we performed a correlation analysis between brain tryptophan and the ratio of plasma tryptophan to the five competing amino acids among individual rats given the diets shown in Fig. 1 (Fig. 2). This analysis gave a correlation coefficient of 0.95 ($P < .001$ that $r = 0$), whereas a correlation of brain tryptophan with plasma tryptophan alone was less striking ($r = 0.66$; $P < .001$ that $r = 0$). Further, a correlation

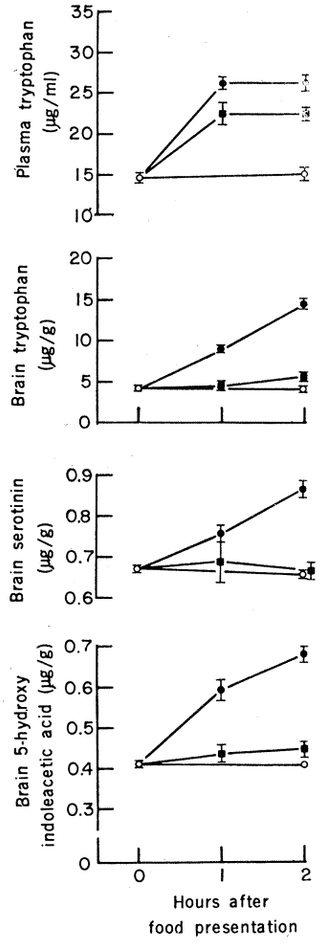

Fig. 1. Effect of the ingestion of various amino acid-containing diets on plasma and brain tryptophan, and brain 5-hydroxyindoles. Groups of eight rats were killed 1 or 2 hours after diet presentation. Vertical bars represent standard errors of the mean. (○—○), Fasting controls; (■—■), complete amino acid–mixture diet; (●—●), mixture diet minus tyrosine, phenylalanine, leucine, isoleucine, and valine. The 1- and 2-hour plasma tryptophan concentrations were significantly greater in animals consuming both diets ($P < .001$) than in fasting controls. All brain tryptophan, serotonin, and 5-hydroxyindoleacetic acid concentrations were significantly greater in rats consuming the diet lacking the five amino acids than in fasting controls ($P < .001$ for all but 1-hour serotonin, $P < .01$). Among animals eating the complete amino acid mixture, the 2-hour brain tryptophan concentration was significantly above that of the corresponding fasting controls ($P < .001$).

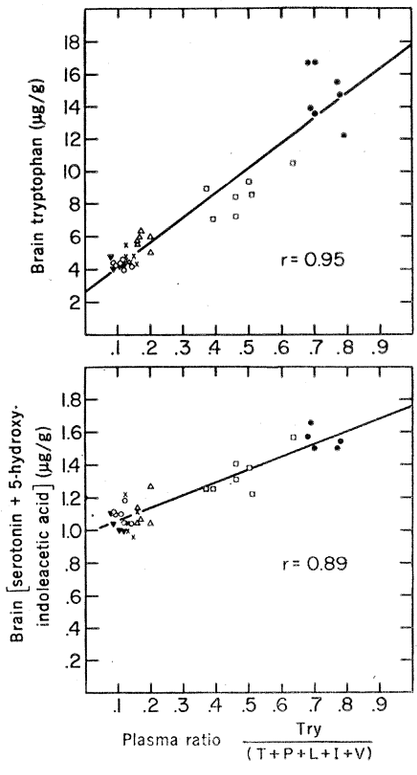

Fig. 2. (A) Correlation between brain tryptophan (*Try*) concentration and the plasma ratio of tryptophan to the five competing amino acids in individual rats studied in the experiment described in Fig. 1 ($r = 0.95$, $P < .001$ that $r = 0$). (B) Correlation between the sum of brain serotonin and 5-hydroxyindoleacetic acid, and the plasma ratio of tryptophan to the five competitor amino acids (*T*, tyrosine; *P*, phenylalanine; *L*, leucine; *I*, isoleucine; *V*, valine) in individual rats studied in the experiment described in Fig. 1 ($r = 0.89$, $P < .001$ that $r = 0$). (○) The 1- and (▼) 2-hour controls; (×) 1-hour complete amino acid mix diet; (△) 2-hour complete amino acid mix diet; (□) 1-hour complete mixture diet minus five competing amino acids; (●) 2-hour complete mixture diet minus five competing amino acids.

between brain 5-hydroxyindoles (serotonin plus 5-hydroxyindoleacetic acid) and the plasma amino acid ratio gave a coefficient of 0.89 ($P < .001$), whereas a correlation with plasma tryptophan alone was less noteworthy ($r = 0.58$; $P < .001$) (Fig. 2). Thus, the brain concentrations of both tryptophan and the 5-hydroxyindoles more nearly reflect the ratio of plasma tryptophan to competing amino acids than the plasma tryptophan alone. Tryptophan in plasma is divided between a larger, albumin-bound pool and a smaller, free pool (*11*). If brain tryptophan is in equilibrium with free rather than total plasma tryptophan, these correlations may be improved even further by substituting free for total trytophan in the plasma ratio (*12*).

The effect of food consumption on brain 5-hydroxyindoles may now be modeled as in Fig. 3. Since carbohydrate ingestion elicits insulin secretion, it simultaneously raises plasma tryptophan and lowers the concentrations of the competing neutral amino acids in rats (*2*); hence, the ratio of plasma tryptophan to competing amino acids increases, leading to elevations in brain tryptophan and serotonin. Protein consumption provides the plasma with a source of all the amino acids; however, the ratio of tryptophan to competitor amino acids in dietary proteins is almost always lower than this ratio in plasma (*5*). Probably for this reason, protein ingestion increases plasma tryptophan less than it does the plasma concentrations of competing amino acids, thereby decreasing the ratio. The insulin secretion elicited by protein consumption will, by itself, produce an opposite change in this ratio. Thus, brain tryptophan and 5-hydroxyindoles could decrease, increase, or remain unchanged after protein consumption, depending on the amino acid composition of the dietary protein, and the proportion of protein to carbohydrates.

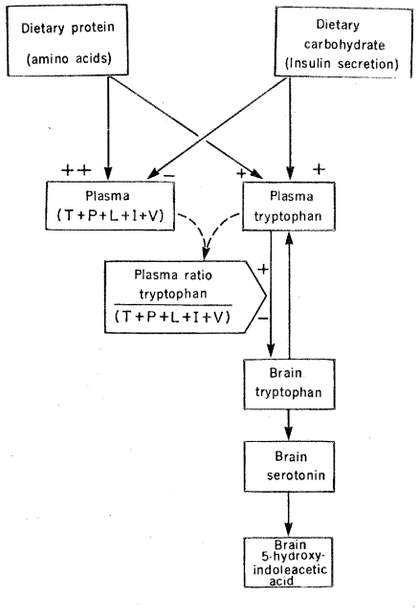

Fig. 3. Proposed sequence describing diet-induced changes in brain serotonin concentration in the rat. The ratio of tryptophan to tyrosine (*T*) plus phenylalanine (*P*) plus leucine (*L*) plus isoleucine (*I*) plus valine (*V*) in the plasma is thought to control the tryptophan concentration in the brain.

Studies of the competition among various amino acids for uptake into the brain have largely utilized brain slice preparations, or animals treated with pharmacologic doses of one or two amino acids (*9, 10, 13*). Our data provide evidence that such competition occurs in the concentration ranges that normally exist in untreated animals consuming natural proteins; further, this action appears to control the content of a putative neurotransmitter, serotonin in the brain. Since a wide variety of dietary and hormonal inputs probably can influence the ratio of plasma tryptophan to its competitor amino acids, our data suggest that serotonin-containing neurons provide the rest of the brain with information about a broad range of metabolic states (*14*).

JOHN D. FERNSTROM
RICHARD J. WURTMAN
Laboratory of Neuroendocrine Regulation, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge

References and Notes

1. J. D. Fernstrom and R. J. Wurtman, *Science* **173**, 149 (1971).
2. ———, *Metabolism* **21**, 337 (1972).
3. ———, *Science* **174**, 1023 (1971).
4. W. Lovenberg, E. Jequier, A. Sjoerdsma, *Advan. Pharmacol.* **6A**, 21 (1968).
5. Composition of amino acid mixture (grams per 100 g of mixture): tryptophan, 1.07; threonine, 4.45; isoleucine, 5.51; leucine, 8.35; lysine, 7.48; methionine, 2.58; cysteine, 0.27; phenylalanine, 4.53; tyrosine, 5.69; valine, 6.57; arginine, 3.74; histidine, 2.85; alanine, 2.94; aspartate, 6.48; glutamate, 20.04; glycine, 1.78; proline, 9.70; and serine, 5.70. Mixture taken from M. L. Orr and B. K. Watt, *Home Economics Research Report No. 4* (U.S. Department of Agriculture, Washington, D.C., December 1957).
6. W. D. Denckla and H. K. Dewey, *J. Lab. Clin. Med.* **69**, 160 (1967).
7. J. H. Thompson, Ch. A. Spezia, M. Agnulo, *Experientia* **26**, 327 (1970).
8. B. T. Ho and D. Taylor, *Biochem. Med.* **5**, 521 (1971).
9. R. Blasberg and A. Lajtha, *Arch. Biochem. Biophys.* **112**, 361 (1965).
10. G. Guroff and S. Udenfriend, *J. Biol. Chem.* **237**, 803 (1962).
11. R. H. McMenamy and J. L. Oncley, *ibid.* **233**, 1436 (1958).
12. It has recently been shown that in human subjects carbohydrate ingestion causes a selective decline in free (that is, nonalbumin-bound) plasma tryptophan, coincident with insulin-mediated reduction in plasma free fatty acids (D. Lipsett, B. K. Madras, R. J. Wurtman, H. N. Munro, in preparation).
13. A. Yuwiler and R. J. Louttit, *Science* **134**, 831 (1961); A. Yuwiler and E. Geller, *Nature* **208**, 83 (1965); S. Roberts, *Progr. Brain Res.* **29**, 235 (1968).
14. The precise uses to which the brain puts this information await identification. However, in collaboration with Dr. Loy Lytle, we have obtained some evidence that the increase in brain serotonin induced by an injection of L-tryptophan is associated with dose-related decreases in food consumption and motor activity. Similar injections of D-tryptophan or L-lysine, which do not increase brain serotonin, fail to modify food consumption and activity.
15. Supported in part by grants from the John A. Hartford Foundation and the National Aeronautics and Space Administration (NGR-22-009-627).

6 June 1972