Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, 2nd ed, Springfield, Ill., Thomas, p 74.
Landsteiner, K. (1936), The Specificity of Serological Reactions, Springfield, Ill., Thomas.
Mills, J. A., and Haber, E. (1963), *J. Immunol. 91*, 536.
Orachterlony, O. (1948), *Acta Path. Microbiol. Scand. 25*, 186.
Potts, J. T., Jr., Young, D. M., and Anfinsen, C. B. (1963), *J. Biol. Chem. 238*, 2593.
Richards, F. M., and Vithayathil, P. J. (1959), *J. Biol. Chem. 234*, 1459.
Scheraga, H. A., and Rupley, J. A. (1962), *Advan. Enzymol. 24*, 161.
Sherwood, L. M., and Potts, J. T., Jr. (1965a), *J. Biol. Chem. 240*, 2799.
Sherwood, L. M., and Potts, J. T., Jr. (1965b), *J. Biol. Chem. 240*, 3806.
Singer, S. J., and Richards, F. M. (1959), *J. Biol. Chem. 234*, 2911.
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.

Glutamine Synthetase. Determination of Its Distribution in Brain during Development*

Soll Berl†

ABSTRACT: Glutamine synthetase and glutamyl transferase activity have been assayed in the neocortex, hippocampus, cerebellum, diencephalon, and pons–medulla of developing kittens and mature animals. The method used for synthetase assay enabled the determination of enzyme activity in homogenates of whole brain tissue. The five brain areas showed different patterns of development for the enzyme. The neocortex had the sharpest increase in enzyme activity and achieved the highest values. In the neocortex the rate of enzyme development coincided with the pattern of development of the compartmentation of glutamic acid metabolism (Berl, 1965); this was not true for the other brain areas (Berl and Purpura, 1965).

The presence in brain of metabolic compartments or pools of glutamic acid which are not in rapid equilibrium with each other has been established by several investigations (Berl *et al.*, 1961 Berl *et al.*, 1962a; 1962b; Waelsch *et al.*, 1964). However, evidence for a separate pool of glutamic acid preferentially used for glutamine formation appears in various parts of the brain at different periods during ontogenesis (Berl, 1965; Berl and Purpura, 1965). Since glutamine synthetase activity may contribute to the emergence of the glutamic acid–glutamine compartmentation system, the changes in levels of this enzyme in the neocortex (cerebral cortex), hippocampus, cerebellum, diencephalon, and brain stem (pons–medulla) of the postnatal animal was followed to determine the extent of its coincidental or correlative development with the development of such compartmentation.

The determination of glutamine synthetase activity in brain tissue homogenates is hampered by the inhibitory effect of adenosine diphosphate (ADP) (Elliott, 1951). It has also been described that the presence of adenosine triphosphate (ATP) and Mg^{2+} offers at least 85% protection against enzyme inactivation at 60° for 10 min (Pamiljans, *et al.*, 1962). A method is herein described which takes advantage of the latter finding for the inactivation of ATPase in an assay for glutamine synthetase activity in whole brain tissue. Glutamyl transferase activity was determined for comparison as an aid in the evaluation of the method; there is little doubt that glutamine synthetase and glutamyl transferase activities are associated with the same enzyme (Meister, 1962). The activity of the enzyme in sixteen brain areas of the adult cat are also reported.

Methods

The glutamine synthetase method is based on the elimination of the inhibitory effect of ADP. This is accomplished by heat inactivation of ATPase (10 min at 58–60°). The addition of ATP and Mg^{2+} protects the synthetase enzyme from inactivation (Pamiljans *et al.*,

* From the Department of Neurological Surgery, Columbia University, College of Physicians and Surgeons, New York, New York. *Received October 22, 1965.* This research has been supported in part by a grant (NB-04064-03) from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service, and the Paul Moore Neurosurgical Research Fund.

† Research Career Development Awardee of the National Institute of Neurological Diseases and Blindness, National Institute of Health (5K-3NB-5117-03).

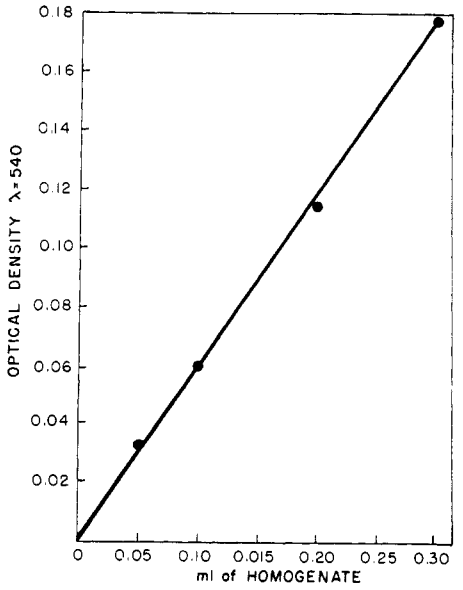

FIGURE 1: Glutamine synthetase assay of rat cerebral cortex; conditions as described in the text, incubation time = 15 min.

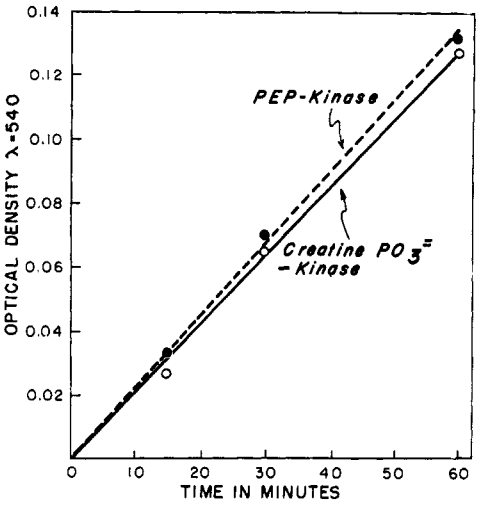

FIGURE 2: Time curve. Glutamine synthetase assay of kitten cerebral cortex (2 weeks old). ATP regenerating systems were phosphoenol pyruvate–pyruvate kinase (PEP–Kinase) and creatine phosphate–creatine phosphokinase (Creatine PO_3^{2-}–Kinase). Conditions as described in the text; 0.2 ml of 1:10 homogenate of tissue in H_2O.

1962). An ATP regenerating system consisting of phosphoenol pyruvate and pyruvate kinase or creatine phosphate and creatine phosphokinase is then added for the conversion of the ADP to ATP.

The assay system is based on that used by Pamiljans *et al.* (1962). The pyruvate kinase (0.05 mg), 2-phosphoenol pyruvate tricyclohexylammonium salt (0.01 M final concentration), hydroxylamine-HCl (pH 7.2, freshly neutralized), and the L-glutamate Na salt (pH 7.2) are added after the heat treatment. The tissue is homogenized with ten volumes of H_2O and 0.1, 0.2, and 0.3-ml aliquots are assayed. The final volume is 1.0 ml.

The mixture is then incubated at 37° for 15 min or longer if necessary. The reaction is stopped by the addition of 1.5 ml of 10% $FeCl_3$ in 0.5 N HCl containing 8% trichloroacetic acid. The mixture is clarified by centrifugation. The absorbance at 540 mμ is measured. One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmole of γ-glutamylhydroxamate/hr. Synthetic γ-glutamylhydroxamate was used as a standard.

The glutamyl transferase assay was carried out in the system described by Rudnick *et al.* (1954). Similar aliquots of the tissue homogenates used for the synthetase assay were also used for the transferase assay. The final volume was 1.0 ml.

ATPase activity was determined by the release of phosphate from ATP under the conditions of the synthetase assay procedure. Phosphate was determined by the method of Fiske and Subbarow (1925). Kittens ranging in age from 2 days to 13 weeks and adult animals were studied. Pentobarbital was used as a general anesthetic and the brain quickly removed, dissected, and the tissue placed in 0.9% saline in an ice bath. The tissue was subsequently blotted on

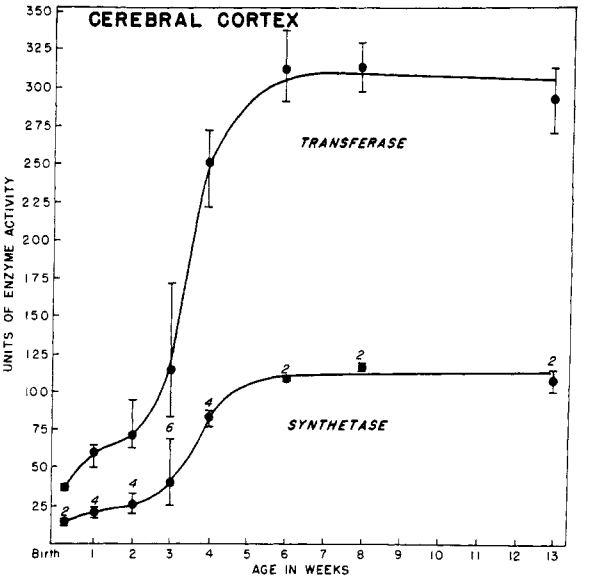

FIGURE 3: Glutamine synthetase and transferase activity of kitten cerebral cortex. Units of enzyme activity/gram of tissue wet weight are given. 1 unit = 1 μmole of γ-glutamylhydroxamate formed/hr. The range of values, the average value, and the number of animals for each point are indicated.

filter paper to remove the saline, weighed, and homogenized with ice-cold water. The homogenates were kept in ice until assayed. Protein was determined in diluted aliquots of the tissue homogenates by the method of Lowry *et al.* (1951). Bovine serum albumin served as the standard.

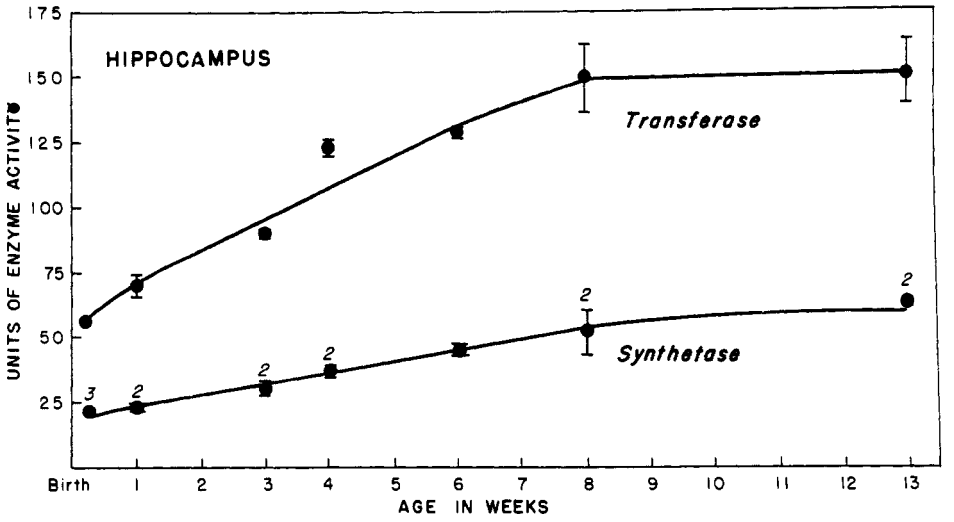

FIGURE 4: Kitten hippocampus. See Figure 3.

TABLE I: Glutamine Synthetase and Transferase Activity in the Adult Cat Brain.

Brain Areas	Synthetase		Transferase		Ratio Transferase/ Synthetase	Protein (%)
	Units[a]/g of Tissue	Units/mg of Protein	Units/g of Tissue	Units/mg of Protein		
Sensory–motor cortex	123.2	1.47	346.8	3.85	2.81	9.0
Auditory cortex	113.6	1.21	314.0	3.34	2.76	9.4
Visual cortex	112.7	1.21	320.5	3.45	2.84	9.3
Association cortex	100.5	1.05	314.3	3.27	3.13	9.6
Quadrigeminal plate	81.6	0.85	247.1	2.57	3.03	9.6
Caudate nucleus	72.9	0.77	220.3	2.32	3.02	9.5
Hippocampus	71.3	0.74	186.9	1.95	2.62	9.6
Thalamus	65.2	0.66	183.0	1.85	2.81	9.9
Hypothalamus	64.4	0.76	175.8	2.07	2.73	8.5
Lateral geniculate body	64.4	0.72	196.2	2.18	3.05	9.0
Anterior cerebellum	65.1	0.71	191.6	2.08	2.94	9.2
Cerebellar hemispheres	53.3	0.57	175.5	1.91	3.29	9.2
Midbrain tegmentum	52.3	0.64	151.6	1.85	2.90	8.2
Pons	53.2	0.65	151.1	1.84	2.84	8.2
Medulla	48.3	0.60	145.3	1.79	3.01	8.1
Corpus callosum	34.5	0.43	121.8	1.50	3.53	8.1
Av ± std dev					2.96 ± 0.23	

[a] 1 unit = 1 μmole of γ-glutamylhydroxamate formed/hr. Each value is an average of the data obtained from two animals.

Results

Under the conditions of the synthetase assay system in 15 min 2.12 μmoles of phosphate was released from the ATP by 0.3 ml of a 1:10 homogenate of cerebral cortex in water as compared with 0.16 μmole following heat treatment at 60° for 10 min, a reduction of ATPase activity of 92.5%.

Whereas without ATPase inactivation synthetase activity is not proportional to the amount of tissue assayed, with heat treatment and the subsequent addition of an ATP regenerating system to the assay system such proportionality is obtained (Figure 1). Phosphoenol pyruvate plus pyruvate kinase may be slightly better in the assay than creatine phosphate plus creatine phosphokinase, although both maintain the enzyme activity undiminished for at least an hour (Figure 2).

The developmental data for synthetase and transferase activity obtained from the kittens are given in Figures

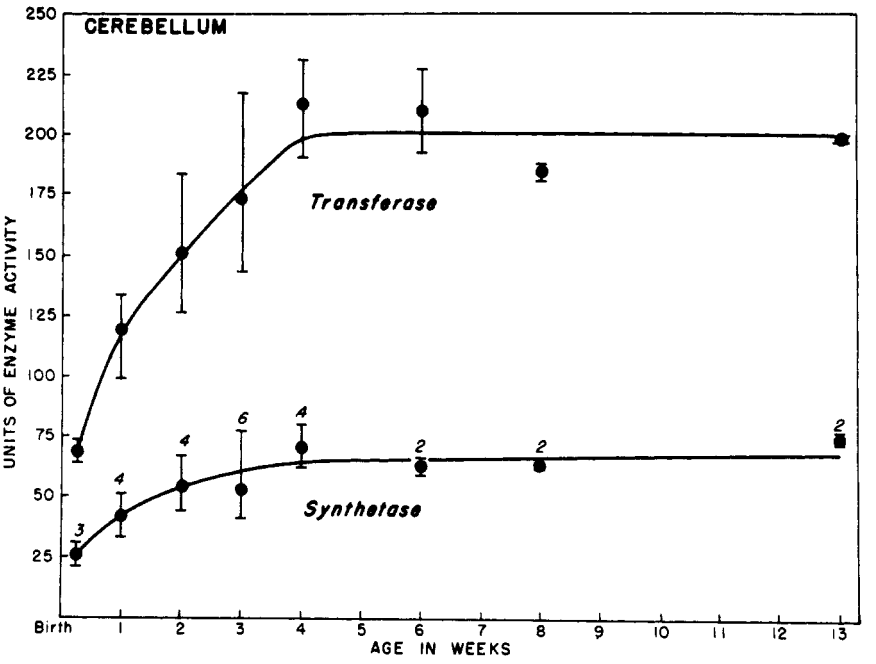

FIGURE 5: Kitten cerebellum. See Figure 3.

3 through 7. In the neocortex (Figure 3) the enzyme activity shows a four-fivefold increase between the first 2 days and the sixth week of life, the greatest increase occurring between the second and fourth postnatal week. In the hippocampus (Figure 4), a part of the archicortex, the increase in enzyme activity is much more gradual and reaches values only approximately half that of the neocortex at 8 weeks of age. The same is true for the cerebellum (Figure 5) although the rate of increase appears to be somewhat greater than in the hippocampus, for the high point of the activity curve appears to be reached at approximately 4 weeks of age. In the meso-diencephalon (Figure 6) and brain stem (Figure 7) the enzyme level is somewhat higher in the neonatal animal and its increase is less than in the other brain areas. In the former there is no apparent change in enzyme activity during the first 2 weeks followed by a small increase thereafter. In the latter there occurs a small rise during the first week followed by an equal decline in enzyme activity during the next 2 to 3 weeks and a slight rise again during the ensuing 2 to 3 weeks. The transferase and synthetase curves have quite similar contours and their ratio is approximately 3 in all areas.

The glutamine synthetase and glutamyl transferase activity in 16 brain areas of the adult cat are described in Table I. The neocortex has the highest enzyme activity. The hippocampus has considerably less activity and so does the meso-diencephalic area. The cerebellum, pons, and medulla contain approximately the same amount of enzyme activity and are at the lower end of the scale. The corpus callosum exhibits the least activity, approximately one-third of that of the neocortex. The above is true whether enzyme activity is calculated for the tissue on a wet weight basis or per milligram of protein.

The ratio of transferase to synthetase activity is again approximately 3 with little significant variation from area to area. Data on the protein content of the several areas of the developing brain were obtained and some of the data from Figures 3–7 were recalculated as units/milligram of protein (Table II). These results, although incomplete, indicate only slight variations in levels and developmental patterns for the protein in these areas. Therefore the patterns of enzyme development which emerge are about the same whether calculated on a wet weight basis or per milligram of protein.

Discussion

Since glutamine synthetase is inhibited by ADP, the enzyme activity depending upon the ratio of ATP to ADP (Elliott, 1951), it has not been possible heretofore to assay for the enzyme in whole brain tissue homogenates. This procedure did become possible with modification, as described in this paper, of methods applicable to purified or partially purified preparations.

In the present study the ratio of transferase to synthetase activity shows no significant variation from area to area, and this is to be expected. Although this ratio does vary considerably with the source of enzyme (Levintow *et al.*, 1955), the ratio reported here is in keeping with the results obtained by Rudnick and Waelsch (1955) in chick brain and retina.

A consideration of the differences in enzyme activity between corpus callosum and neocortex (Table I) may be of interest. Deoxyribonucleic acid (DNA) determinations indicate that most brain areas, including the

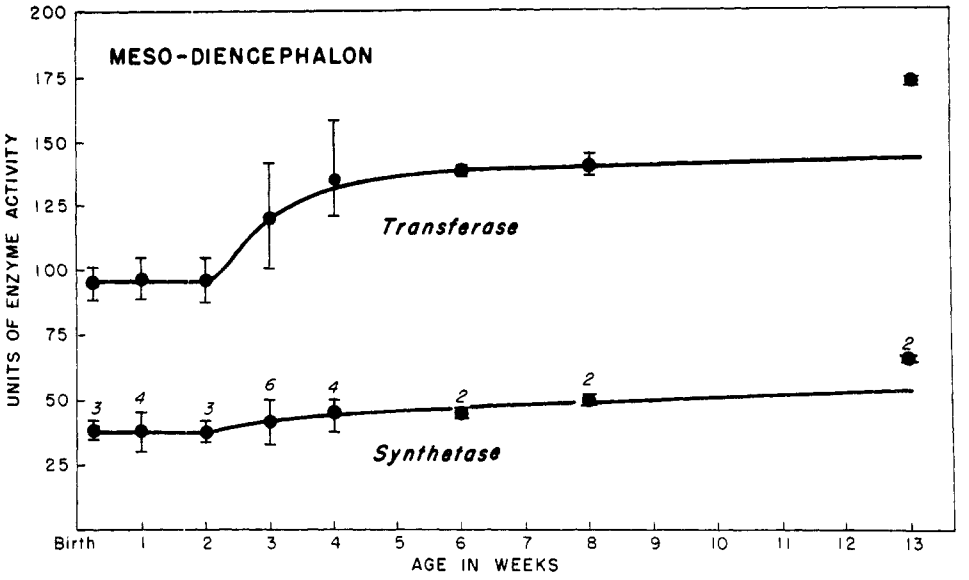

FIGURE 6: Kitten diencephalon. See Figure 3.

TABLE II: Per Cent Protein and Glutamine Synthetase Activity of Cat Brain.

	% Protein					Glutamine Synthetase[a] (units/mg of Protein)				
Age	Cerebral Cortex	Hippocampus	Cerebellum	Mesodiencephalon	Brain Stem	Cerebral Cortex	Hippocampus	Cerebellum	Mesodiencephalon	Brain Stem
One day[b]	6.0	6.2	6.4	6.3	6.7	0.22	0.30	0.31	0.58	0.37
Two days	6.3		7.3	6.7	7.0	0.23		0.34	0.55	0.42
One week[c]	6.8	6.9	7.5	6.9	7.4	0.31	0.34	0.53	0.53	0.52
Three weeks	7.1	7.0	8.3	7.6	7.8	0.59	0.45	0.72	0.55	0.40
Five weeks	8.0	7.8	9.0	7.6	8.4	1.25	0.51	0.70	0.61	0.41
Adult[d]	9.3	9.6	9.2	9.1	8.2	1.24	0.74	0.64	0.73	0.63

[a] Kitten values based on the data in Figures 3–7. [b] Average of two animals. [c] Average of three animals. [d] Obtained from Table I.

corpus callosum, contain approximately the same number of cells per unit weight; the cerebellum on the other hand has many more cells (Heller and Elliott, 1954; Mihailović, *et al.*, 1958). While the neocortex is rich in neuronal cells, the ratio of glial to neuronal cells being about 1.5 as described by Brizzie and Jacobs (1959), the corpus callosum contains mainly glial cells or their derivatives. Therefore the fact that the cortex has approximately three times greater enzyme activity than the corpus callosum suggests that neuronal cells have many times greater capacity for glutamine formation than glial cells, if one can assume that the glial cells of the corpus callosum are similar to those in the cortex. Lower enzyme activity in the other brain areas may result, therefore, because the neuronal cells in these areas have less enzyme activity than those of the neocortex or the ratio of glial cells to neuronal cells is greater. Other than in the neocortex there are no data on the ratio of glial to neuronal cells. One must also bear in mind that any area of brain contains many different types of neuronal cells. It is possible that the distribution of the enzyme may vary considerably according to cell type.

The main purpose of these studies was to determine the extent of participation of glutamine synthetase in the development of the glutamic acid–glutamine compartmentation system in several brain areas and further whether evidence could be obtained for its contribution to the morphophysiological development of this organ.

Several points may be made. At the time of birth of the kitten, the neocortex, hippocampus, and cerebellum have attained different stages of development. Neurons in the hippocampus appear to be about 2 weeks more advanced in structure and physiological properties than those of the neocortex (Purpura, 1964). In contrast, the Purkinje cells of the cerebellum are about 2

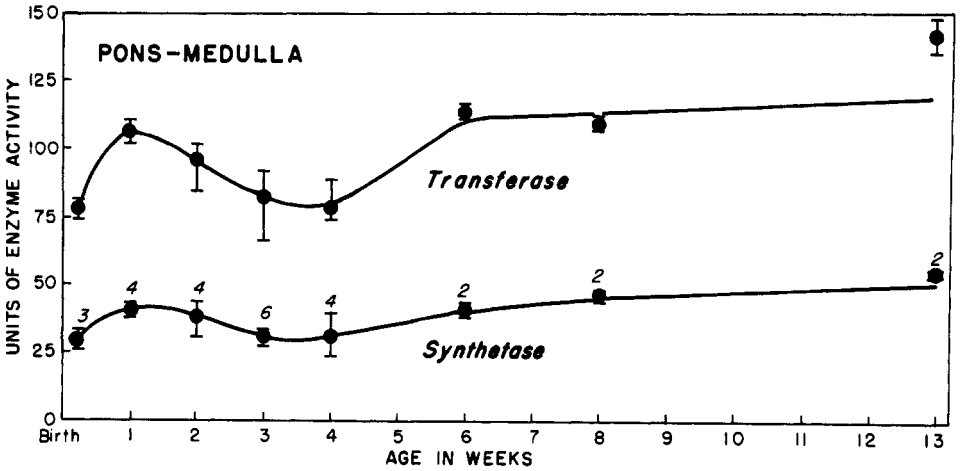

FIGURE 7: Kitten pons–medulla. See Figure 3.

to 3 weeks less developed than the major cell types of the neocortex (Purpura *et al.*, 1964). Yet in these three areas, the enzyme is at about the same low level shortly after birth. Therefore glutamine synthetase capacity does not appear to have any relationship to the early initial maturation of these neuronal cells. Particularly in the neocortex and hippocampus, glutamine synthetase activity does not correlate well with early postnatal neuronal ontogenesis. Whether such a correlation exists during prenatal stages in development when the enzyme activity is present at relatively lower concentrations remains to be determined. Its increasing activity appears to be more related in time to the increasing volume of these areas which in turn is accounted for mainly by dendritic and concomitant synaptic elaboration and glial proliferation (Purpura *et al.*, 1964).

In the chick central nervous growth of synthetase–transferase activity also occurs late in the development of the nervous system as compared with general protein growth. In the retina, however, there is a sharp rise in enzyme activity which strongly suggests correlation with visual maturation (Rudnick and Waelsch, 1955). These authors suggest that the activity of this enzyme may be related to functional maturation of particular types of neuronal cells. Similar results were obtained in tissue culture of chick retina (Mascona and Hubby, 1963).

The increase in enzyme and the development of the glutamic acid–glutamine system of compartmentation also do not correlate well in all areas. Only in the neocortex does the increase in enzyme activity coincide with that of the development of compartmentation of glutamic acid metabolism (Berl, 1965); both occur mainly during the third to the sixth postnatal weeks. In the hippocampus such compartmentation is already evident at 2 days of age (Berl and Purpura, 1965) at a relatively low enzyme level while in the cerebellum, midbrain, and brain stem evidence for compartmentation appears at a time of achievement of maximum or close to maximum levels of enzyme activity. It seems, therefore, that glutamine synthetase activity is only one factor in the compartmentation of glutamic acid metabolism; anatomical factors may require equal consideration. A large part of the enzyme is associated with the microsomes (Waelsch, 1959) or the endoplasmic reticulum (Sellinger and Verster, 1962), a small amount may also be associated with the mitochondria, and part of it is always found unassociated in the cytoplasm. A correlation of structure with enzyme activity suggests that the ratio of distribution of the enzyme among the various cellular fractions or parts of cells, *e.g.*, synaptic endings (Salganicoff and De Robertis, 1965), may be of greater importance than total enzyme activity.

Acknowledgment

The author wishes to express his gratitude to Dr. Dominick P. Purpura for his help in the preparation and dissection of the animals, and to Miss Iren Tar and Mr. Ramiro Nunez for their excellent technical assistance.

References

Berl, S. (1965), *J. Biol. Chem. 240*, 2047.

Berl, S., Lajtha, A., and Waelsch, H. (1961), *J. Neurochem. 7*, 186.

Berl, S., and Purpura, D. P. (1965), *J. Neurochem.* (in press).

Berl, S., Takagaki, G., Clarke, D. D., and Waelsch, H. (1962a), *J. Biol. Chem. 237*, 2562.

Berl, S., Takagaki, G., Clarke, D. D., and Waelsch, H. (1962b), *J. Biol. Chem. 237*, 2570.

Brizzie, K. R., and Jacobs, L. A. (1959), *Anat. Record 134*, 97.

Elliott, W. H. (1951), *Biochem. J. 49*, 106.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Heller, I. H., and Elliott, K. A. C. (1954), *Can. J. Bio-*

chem. Physiol. 32, 584.
Levintow, L., Meister, A., Hogeboom, G. H., and Kuff, E. L. (1955), *J. Am. Chem. Soc. 77*, 1955.
Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Meister, A. (1962), *in* The Enzymes, Vol. 6, Boyer, P. D., Lardy, H., and Myrbäck, K., Eds., New York, N. Y., Academic, p 443.
Mihailović, Lj., Janković, B. D., Petković, M., and Mancić, D. (1958), *Experientia XIV*, 9.
Moscona, A. A., and Hubby, J. L. (1963), *Develop. Biol. 7*, 192.
Pamiljans, V., Krishnaswamy, P. R., Dumville, G., and Meister, A. (1962), *Biochemistry 1*, 153.
Purpura, D. P. (1964), *in* Neurological and Electroencephalographic Correlative Studies in Infancy, Kellaway, P., Ed., New York, N. Y., Grune and Stratton, p 117.
Purpura, D. P., Shofer, R. J., Housepian, E. M., and Noback, C. R. (1964), *in* Progress in Brain Research. Growth and Maturation of the Brain, Vol. 4, Purpura, D. P. and Schadé, J. P., Eds., Amsterdam, Elsevier, p 187.
Rudnick, D., Mela, P., and Waelsch, H. (1954), *J. Exptl. Zool. 126*, 297.
Rudnick, D., and Waelsch, H. (1955), *J. Exptl. Zool. 129*, 309.
Salganicoff, L., and De Robertis, E. (1965), *J. Neurochem. 12*, 287.
Sellinger, O. Z., and Verster, F. DeB. (1962), *J. Biol. Chem. 237*, 2836.
Waelsch, H. (1959), *in* Proceedings of the Fourth International Congress of Biochemistry, Vol. 3, Vienna, 1958, London, Pergamon, p 36.
Waelsch, H., Berl, S., Rossi, C. A., Clarke, D. D., and Purpura, D. P. (1964), *J. Neurochem. 11*, 717.

In Vivo Formation of Tyrosine from *p*-Fluorophenylalanine*

Gail Dolan and Claude Godin

ABSTRACT: When DL-phenylalanine-3-^{14}C and DL-fluorophenylalanine-3-^{14}C are injected into rats, tyrosine-^{14}C is found incorporated into both plasma and pancreas proteins. No phenylalanine-^{14}C derived from fluorophenylalanine-^{14}C is found in proteins. The percentage of the protein radioactivity derived from tyrosine-^{14}C is higher when fluorophenylalanine is injected than when phenylalanine is injected. This would indicate a fast transformation of fluorophenylalanine into tyrosine.

p-Fluorophenylalanine, an analog of phenylalanine, is known to be incorporated into bacterial and mammalian proteins both *in vivo* and *in vitro* (Fruton, 1963). This is explained by its ability to replace phenylalanine in protein peptide chains. However, fluorophenylalanine is known to be very toxic to rats when added to the diet (Armstrong and Lewis, 1951a). This toxicity was attributed to the formation of fluoride ions (Armstrong and Lewis, 1951b).

Recently Kaufman (1961, 1964) has reported that, *in vitro*, fluorophenylalanine is transformed directly into tyrosine at one-sixth the rate of the conversion of phenylalanine to tyrosine. This reaction, which is catalyzed by the enzyme phenylalanine hydroxylase, produces equal amounts of L-tyrosine and fluoride ions. No phenylalanine is formed, and only synthetic pteridine derivatives can act as the cofactor in the transformation.

In our laboratory we are studying the metabolism of phenylalanine-3-^{14}C and fluorophenylalanine-3-^{14}C in rats. We have obtained incorporation of fluorophenylalanine into tissue proteins and wish to report evidence that the radioactive tissue proteins isolated contained two radioactive amino acids, tyrosine and fluorophenylalanine.

Experimental Section

DL-Phenylalanine-3-^{14}C (1 μc/mg) and DL-*p*-fluorophenylalanine-3-^{14}C (1 μc/mg) were injected intravenously to albino Wistar rats weighing about 150 g. Each rat received 1 mg of the radioactive amino acid in 0.5 ml of isotonic NaCl. The rats were killed by decapitation after 2, 6, and 24 hr. The blood was collected and certain organs were removed. The trichloroacetic acid insoluble proteins were obtained from the plasma and the pancreas after homogenization. The dry insoluble proteins were hydrolyzed under vacuum in 6 N HCl at 100° for 18 hr. The hydrolysates were analyzed

* From the Department of Biology, University of Ottawa, Ottawa, Canada. *Received November 17, 1965.* Supported by the National Research Council of Canada. Paper presented at the 150th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1965.