Protein Degradation Rates in Regions of the Central Nervous System *in vivo* during Development

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The rate of protein degradation was estimated in several regions of rat brain at various ages by subtracting the rate of accumulation of protein from the rate of synthesis. The rate of degradation in cerebral hemisphere, which was 1.3%/h at 2 days of age, declined steadily with age, approaching the synthesis rate at about 30 days of age (0.8%/h). Degradation rates in the pons medulla, mid-brain and spinal cord were of a similar order to that in the cerebral hemisphere. The cerebellum had an exceptionally high rate of degradation in young rats, 1.9%/h at 2 days of age, which complemented its high rates of synthesis and accumulation. The degradation rate in the young was 2–2.5 times the rate in older rats and was approx. 65% of the synthesis rate during the more active phase of growth. The rapid accumulation of protein in the nervous system during the first week *post partum* was accompanied by high rates of breakdown, and was the result of a relatively small difference between that high rate of degradation and an even higher synthesis rate.

Protein metabolism during development of the nervous system has been of considerable interest, in part because various disorders often associated with mental retardation, such as phenylketonuria and malnutrition, are suspected of operating through derangements of synthesis or degradation of brain protein. Although synthesis measurements have been the subject of many studies, there are virtually no rate measurements of protein degradation *in vivo* in the brain. The dearth of information on this subject is due to the absence of suitable procedures for determining rates. There are numerous methods applicable to individual proteins, but none for the rate of the overall process in brain.

Degradation rates can, however, be computed indirectly from changes in the net synthesis rate if the total synthesis rate is known. In the adult animal, where the total brain protein content is practically constant, one can merely assume that degradation must nearly equal synthesis. But during development the large changes in total protein can be used along with synthesis rate measurements to estimate, within limits, the average rate of degradation.

The accuracy of this approach depends directly on the measurements of the protein-synthesis rate and the rate of accumulation of protein. We have used a method for measuring synthesis rates that involves injecting large quantities of labelled valine as protein precursor (Dunlop *et al.*, 1975a). This procedure establishes a constant precursor specific radioactivity during the incorporation period and has provided protein-synthesis rate measurements in various

regions of rat brain at different ages (Dunlop *et al.*, 1977) that are more extensive and exhibit relatively smaller variations than the few quantitative rate measurements previously available from pulse-labelling experiments (e.g. Oja, 1967).

In the present paper the accumulation rates of protein in the same regions of the nervous system are determined and used, in conjunction with the synthesis rate data, to calculate degradation rates of protein in the rat nervous system at different ages.

Methods

At birth, litters of Wistar rats were decreased where necessary to a maximum of 12 pups. Each litter was maintained in a separate cage with food and water constantly available. Lights were on from 06:00 h to 18:00 h.

Protein-synthesis rates

Synthesis rates were measured by the method of Dunlop *et al.* (1975a). Aqueous solutions of 500 mM-valine containing L-[1-¹⁴C]valine (New England Nuclear Corp., Boston, MA, U.S.A.), usually at 1 μ Ci/ml (sp. radioactivity 4000 d.p.m./ μ mol), were injected intraperitoneally at a dose of exactly 3% of body weight (15 μ mol/g). Rats that were 11 days old or younger were placed in a plastic container in an incubator at 38°C for 30–60 min before injection and replaced immediately after injection. At 30 or 60 min after injection, animals were decapitated and the brains quickly removed and dissected.

For the 'pons medulla' region, the brain was inverted on a flat surface and severed between the cord and medulla and again entering just rostral to the pons, emerging rostral to the anterior colliculus. Of the remaining brain, the outer layers were reflected and taken as 'cerebral layer', and the core was termed 'mid-brain'. In older animals, where striatum could be discerned, it was left with the core. This separation is admittedly imprecise, particularly in the young.

These parts were then homogenized in approx. 5 vol. of 3% (w/v) sulphosalicylic acid. The homogenate was centrifuged at 10000g for 6 min and the supernatant removed. The pellet was resuspended in 2 ml of 5% (w/v) trichloroacetic acid, re-centrifuged, resuspended in the same volume of fresh trichloroacetic acid, heated at 90°C for 15 min and re-centrifuged. After the supernatant was removed the pellet was resuspended in 5 ml of methanol, centrifuged, resuspended in 2 ml of chloroform/methanol (1:1, v/v), centrifuged and finally washed twice in 2 ml of diethyl ether. The dry pellet was weighed.

As an alternative procedure, the brain was chopped (0.25 mm thick) and the slices placed in a scintillation vial containing 5 ml of 3% sulphosalicylic acid. A carton containing up to 100 vials was then placed on a shaker for 15 min. After the sulphosalicylic acid was drawn off, 5 ml of 5% trichloroacetic acid was added to each vial. Thereafter the procedure was as with homogenates, except that an additional chloroform/methanol extraction was inserted, only the slices were shaken in 5 ml of each solvent. The specific radioactivity of the protein (d.p.m./mg of protein residue) processed in this way was 110% of that prepared with the homogenization procedure, and this procedure, being more rapid, was generally used.

Samples (0.10 ml) of the sulphosalicylic acid extract were diluted with water to 2.4 ml and counted for radioactivity with 12 ml of scintillation mixture (Patterson & Greene, 1965) in a Packard Tri-Carb liquid-scintillation spectrometer. Specific radioactivities were determined with a Technicon amino acid analyser with split-stream column effluent. Fractions collected were counted for radioactivity as above.

The weighed protein was dissolved in 2 ml of 1 M-NaOH by heating the vials in boiling water almost to dryness (about 35–40 min). Generally 3–15 mg was counted for radioactivity. To acidify the sample, 0.5 ml of acetic acid was added, followed by 15 ml of scintillation fluid prepared as described by Blasberg & Lajtha (1965). Otherwise 3–12 mg of protein was sealed under vacuum with 5 ml of 6 M-HCl in test tubes and heated at 110°C for 24 h. After drying under air, the hydrolysate was dissolved in 0.2 M-Li⁺/0.05 M-citrate buffer, pH 2.2, and treated in the same manner as the sulphosalicylic acid extracts for

determination of radioactivity or specific radioactivity. Counting efficiency determined by the addition of [¹⁴C]toluene as internal standard was 65% for both scintillators.

In some cases several rats were studied at one age and their littermates at a later time. Although littermates may be compared in this way, the size of the litter was decreased during the period of growth. However, decreasing the litter size from 12 to 6 pups did not change protein-synthesis rates in the brain (Dunlop *et al.*, 1977).

Although there have been reports that high concentrations of some amino acids inhibit brain protein-synthesis rates, we have detected no such effect under the conditions described in the present paper (Dunlop *et al.*, 1975a).

Total protein for protein accumulation rates

Total protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. To minimize errors, each sample of homogenized tissue was diluted on the basis of the expected approximate protein content to about the same final concentration. Thus a very limited segment of the calibration curve was used in these assays.

Protein degradation rates

The degradation rate may be calculated from a mass balance equation

$$dP/dt = K_1P - K_dP \quad (1)$$

where K_d is the degradation constant, K_1 is the fractional synthesis rate, P is the amount of protein and t the time. This approach has been used by Turner & Garlick (1974) to measure K_d in denervated rat diaphragm and by Millward *et al.* (1975) for growing skeletal muscle. Although K_1 can be measured easily at any point, i.e. within 1 h, changes in total protein occur at a much lower rate even in the most rapid stages of brain growth. Also it is necessary to measure relatively small differences between two large quantities of protein derived from different animals. The method is therefore useful only to measure an average K_d over an interval of a day or so.

It is assumed that $K_1 - K_d$ is practically constant and equal to the fractional rate of deposition or accretion of protein (K_A) over an interval of a few days. Thus from eqn. (1), the degradation rate is calculated as:

$$K_d = K_1 - K_A = K_1 - \Delta P / (P \Delta t) \quad (2)$$

Results

Rate of deposition of protein

The increase in total protein for each brain region is shown in Fig. 1. The parameter of interest for calculating K_d is the slope, $\Delta P / \Delta t$, which in general

Fig. 1. Total protein in several regions of nervous tissue during development

Protein was determined by the method of Lowry *et al.* (1951). The average weight of adult rats was 260g. Standard deviations are indicated by bars ($n = 3$ to 12). The curves represent the protein content of the cerebral hemisphere (a), the pons medulla (b), the cord (c), the mid-brain (d) and the cerebellum (e).

is easily determined between 12 and 18 days where the plots approximate straight lines. When the data are plotted as individual litters (results not shown) the various segments of the curves generally give slopes in good agreement with those based on the pooled data.

Both before 10 and after 20 days the slopes are smaller and of changing value. After 30 days the slope is small, except for cord, and the total amount

Fig. 2. Protein accumulation in brain regions in the first 12 days post partum

Protein was determined by the method of Lowry *et al.* (1951). Cerebral hemisphere ($K_A = 0.64\%/h$), $\circ$; pons medulla ($K_A = 0.42\%/h$), $\triangle$; cord ($K_A = 0.56\%/h$), $\blacksquare$; cerebellum ($K_A = 0.92\%/h$), $\bullet$.

of protein is large so that $\Delta P/(P\Delta t)$ becomes insignificant, compared with K_1 , in the calculation of K_A . The solution of eqn. (2) is trivial in such cases, since accretion is practically negligible and hence $K_d = K_1$.

At less than 10 days of age accurate measurement of the slope is difficult, since, as the value is small, a slight difference in the angle introduces a large error. When the data for 2 to 10 days of age are plotted on an expanded scale, a high degree of curvature is evident in most regions (results not shown). In most cases the data were reasonably close to a semi-logarithmic plot, i.e. $\ln(P/P_0) = K_A t$, where the constant K_A is the fractional rate of deposition (Fig. 2). For the mid-brain, the plot of protein content with age was nearly a straight line, which is of course inconsistent with a logarithmic increase in protein. But the mid-brain, as defined above, is the most difficult region to separate, particularly in the young, and this apparent inconsistency may be due to an inadequate separation technique. Fig. 2 also shows that the rate of protein deposition in the cerebellum

Table 1. *Protein accumulation rate in rat brain from 2 to 4 days of age*

Total protein was determined by the method of Lowry *et al.* (1951) in the whole brain and the cerebellum of weight-paired littermates at 2 and 4 days after birth. The linear rate of increase (at 3 days of age) is calculated in %/h as:

$$K_A = \frac{(P_4 - P_2)}{\frac{1}{2}(P_4 + P_2)} \cdot \frac{1}{48}$$

where P_2 and P_4 are the total brain protein at 2 and 4 days of age. For the logarithmic accumulation rate the equation used was $\ln(P_4/P_2) = K_A t$ where $t = 48$ h. Some representative standard deviations are shown ($n = 12$).

	Whole brain		Cerebellum	
	2-day	4-day	2-day	4-day
Wet wt. (mg)	... 330 ± 8	440 ± 20	15.0 ± 1.7	23.6 ± 1.8
Protein content (mg of protein/g wet wt.)	... 66.8 ± 0.7	68.4 ± 0.2	68.7 ± 4.4	72.1 ± 2.8
Total protein (mg)	... 22.2 ± 0.4	30.0 ± 1.23	1.02 ± 0.07	1.68 ± 0.11
K_A (rate of protein increase; %/h)				
Logarithmic		0.62		1.04
Linear (3 days old)		0.61		1.02

is very much higher than in the other regions studied.

Since the anatomical separations were least precise in the young and large variations in total protein were seen, a modified procedure was used with some litters to obtain a reasonably accurate confirmation of K_A at an early age. The rats were weighed each day. At 2 days of age, they were matched according to weight and one of each pair was killed for protein determinations. In half the cases the heavier of each pair was used. Exactly 48 h later, the remaining partner was killed. To minimize separation errors, only the cerebellum and the whole brain were used. The average rate of accretion over 2 days (Table 1) was much higher in the cerebellum (1.04%/h) than in the whole brain (0.62%/h). This may be compared with the values shown in Fig. 2 for the cerebellum (0.92%/h) and the cerebral hemisphere (0.64%/h). The total protein of the whole brain was also calculated from the data in Table 1 of Pitts & Quick (1967), and the rate of accumulation estimated from that curve at 3, 5, 7, 9 and 11 days was 0.56, 0.48, 0.57, 0.58 and 0.43%/h. Although these rates are slightly lower than ours, for the cerebral hemisphere they are reasonably close to a weighted average of the component parts (cerebral hemisphere, mid-brain, pons medulla, plus cerebellum). For comparison, see also the data of Alling & Karlsson (1973).

Protein-synthesis rates

The total rate of protein synthesis (K_1) in these regions has been determined and is shown in Fig. 3 and Table 2 (for details see Dunlop *et al.*, 1977). The rates in cerebral hemisphere, mid-brain, cord and pons medulla are very similar to that in the whole brain, but that for the cerebellum is not. The cerebellum exhibits a synthesis rate fully 50% greater than these other regions in the 2-day-old rat, and that rate remains high over a longer period than for the

Fig. 3. Rates of protein metabolism at different stages of development

Rates of synthesis and accretion were determined as stated in the text. The rate of degradation is the difference between the synthesis and accretion rates. (a) Cerebellum; (b) cerebral hemisphere. ●, Protein-synthesis rate; ■, protein accretion rate; ----, protein degradation rate.

whole brain before decreasing. In an attempt to detect possible circadian rhythms the incorporation rate was estimated in 18-day-old rats at different

Table 2. *Changes in protein-degradation rates with age in regions of the nervous system*

The synthesis-rate measurements were made by the method of injecting large amounts of labelled valine as described in the text (Dunlop *et al.*, 1975a). In calculating degradation rates, two approaches were used. In method A growth rates were determined graphically from expanded curves of the type shown in Fig. 1. Method B is based on the accretion rates determined in Fig. 2.

Age (days) ...	Protein-degradation rates (%/h)					
	2		8		18	37
	A	B	A	B	A	A
Mid-brain						
K_1 (synthesis)	1.86	—	1.43	—	1.13	0.69
K_A (accretion)	1.0	—	0.50	—	0.24	0.03
K_d (degradation)	0.9		0.9		0.9	0.7
Pons medulla						
K_1	1.83	1.83	1.52	1.52	1.24	0.70
K_A	0.6	0.42	0.37	0.42	0.26	0.03
K_d	1.2	1.4	1.1	1.1	1.0	0.7
Cord						
K_1	1.90	1.90	1.64	1.64	1.21	0.71
K_A	0.4	0.57	0.75	0.57	0.32	0.07
K_d	1.5	1.3	0.9	1.1	0.9	0.6

times of the day. Each animal was injected 2h before death with 31 μ mol of L-[1- 14 C]valine/g body wt. After injection, each animal was returned to its cage. The room was dark from 18:00h to 06:00h. The incorporation rate in this one experiment was measured every 4h and ranged from 4.73 ± 0.08 to 4.99 ± 0.13 nmol of valine/h per mg of protein (mean $\pm$ S.D.). In no case were the rates significantly different. Therefore no circadian rhythm was discernible in the whole brain.

Degradation estimates

From the accretion curves (Fig. 1), $\Delta P/(P\Delta t)$ may be determined graphically, or, for ages 2 to 12 days, K_A may be derived directly from Fig. 2 and subtracted from the synthesis rate (K_1) to give the average rate of degradation (K_d). The values of K_d for the cerebellum and the cerebral hemisphere are shown in Fig. 3. Degradation rates at selected ages in the other brain regions are shown in Table 2.

Discussion

There is a large variation between litters in total protein, and this induces a rather wide scatter in the data for young animals. This has occasionally been interpreted as indicating no increase, or only a moderate rate of increase, in total protein for the first 10 days, but, as shown in Table 1, where samples are closely paired and the variation thereby minimized, an accumulation of protein is evident and the rate of increase (%/h) is in fact higher than it is at later ages.

The present results also indicate that the average rate of degradation is higher in the developing

nervous system compared with that of adult rats and, similarly to protein synthesis, rates of breakdown decline rapidly in the first 3 weeks after birth. Thus, at the same stage of development where brain protein is formed at 3 or more times the adult rate, protein is also disappearing at approximately double the rate in adults. The accumulation of protein during this period of rapid growth is therefore the result of a relatively small difference between these highly dynamic processes.

Of the regions studied, the cerebellum stands out as the exception, with considerably higher synthesis, accretion and degradation rates compared with whole brain or the other areas studied here. It is known that the postnatal development of the cerebellum is very different from that of the cerebrum (see Balázs & Richter, 1973). For example, the DNA content of the cerebrum doubles in the first 3 weeks after birth, whereas that of the cerebellum increases 10-fold. Our results show that, not only are the rates of protein metabolism in the cerebellum markedly different from those in other regions, but so also is their course of development; the decline from the maximum rates found in the first few days *post partum* in the rest of the brain is delayed in the cerebellum.

We are not aware of any data in the literature on brain protein-degradation rates *in vivo* obtained by this or other procedures, with which the present results could be compared. Efforts have been made in the past to calculate the rate of degradation from pulse-labelling data, but these have involved the assumption that the specific radioactivity of the protein being degraded was equal to the average specific radioactivity of total protein. It is clear,

however, that after pulse labelling the proteins with short half lives would be most highly labelled and most rapidly degraded. The data of Lajtha *et al.* (1976) indicate that the error inherent in assuming homogeneous turnover of all the proteins after pulse labelling would be very large.

The approach adopted in the present paper has been used by Millward *et al.* (1975) to measure average degradation rates in rat skeletal muscle during development. These workers also found higher rates of synthesis, breakdown and growth at the youngest age studied, and that the rates for all three processes declined with increasing age, though the changes in muscle occur at much later ages and over a much longer interval.

There are data available on the content of proteolytic enzymes in brain, as measured in homogenates, but the pattern of changes in activity with age (Oja & Oja, 1970; Marks *et al.*, 1975) does not follow the rate of protein degradation found here. The content of proteinases increased during the early stages in which our measurements indicate that protein-breakdown rates *in vivo* declined.

Although this approach has enabled us to measure the average rate of degradation at some stages of development, the method obviously has inherent limitations. It is the disappearance rather than degradation of protein that is determined. A portion of this protein could be secreted rather than degraded, but, although there are questions about the quantity and fate of protein transported by axoplasmic flow, there is as yet no evidence that the nervous system secretes any substantial quantity of protein. From the flow rate of cerebrospinal fluid and the protein content of lumbar fluid, it is clear that the loss of protein from adult brain by that route is a tiny fraction of the total disappearance rate. The assumption that degradation and disappearance rates for brain protein are nearly equal therefore appears reasonable. A more serious problem is that the changes in total protein are small, and even with young rats several days, growth is necessary for an accurate measurement. Though the large variations in total protein from litter to litter can be minimized by using weight-paired littermates, short-term perturbations in the rate of degradation would still be extremely difficult to detect. The determination also depends on the assumption that the synthesis rate is more or less constant throughout the period of measurement. In the older animals this seems likely, since the brain temperature probably undergoes a modest circadian rhythm. In the present study we have measured the synthesis rate in 18-day-old rats over a 24 h period and found no significant changes, though of course the animals were awake and agitated at the time of injection. In young animals, which depend on external factors for maintaining body temperature, fluctuations of consequential size and

duration might occur. Brain temperature does profoundly influence synthesis rates (Dunlop *et al.*, 1975b). The average degradation rates are valid only insofar as the brain temperature in the young is maintained close to 37–38°C.

It would be expected that brain protein-synthesis rates should be relatively high during a period of rapid growth. One assumes that the most efficient way of increasing the mass of any protein is to increase K_1 while lowering K_d , and the economics of various processes of this kind have been computed (Paskin & Mayer, 1977). But in brain we find a high rate of degradation and the difference between the processes of synthesis and accretion is large. This presumably implies that a relatively large fraction of the proteins synthesized during development is inherently short-lived or subserves some transient function and is rapidly rendered obsolete. Since 6% of the protein in mature mouse brain accounts for one-half the degradation rate (Lajtha *et al.*, 1976), one may speculate that in the immature nervous system a relatively small highly active fraction might account in large degree for the radically higher rates. This possibility requires investigation.

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